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14. ABSTRACT

The ARF tumor suppressor protein plays an important role in the tumor surveillance of human cancer. In the search for novel ARF binding proteins, we uncovered NPM. Despite the important role ARF plays in the regulation of tumorigenesis, alterations selectively affecting its ability to negate NPM function have not been studied. In our proposed study, we aimed to determine the impact of ARF-NPM interactions in the pathogenesis of breast cancer. To this end, we have found that overexpression of NPM in the absence of ARF is a powerful transforming event. NPM promotes tumorigenesis without affecting genomic stability, implying that the subsequent tumors should remain diploid, a hallmark of ARF-null breast cancers. Indeed, when we analyzed sixty breast carcinomas, NPM was highly overexpressed in 50% of cases. We have begun further analyses of how NPM promotes tumor formation and have discovered that it does so through ribosome dysregulation, opening up the door to new therapeutic targets in breast cancer: protein synthesis.

15. SUBJECT TERMS
Nucleophosmin, ARF, Tumor Suppression, MMTV, Breast Cancer

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INTRODUCTION

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site for active transcription of ribosomal RNAs (rRNA) and ribosome assembly (6). Various nucleolar proteins, RNAs, and other factors have been suggested to be involved in this complex process of ribosome production and maturation (10). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 20). While the nucleolar proteome contains many proteins and ribonucleoproteins proposed to be involved in ribosome biogenesis, a remarkable number of proteins identified (>100) have no known function. The difficulty in assessing nucleolar protein function stems from early assumptions that all nucleolar proteins must be involved, in some way, with static ribosome biogenesis by virtue of their unique subcellular localization. However, a more contemporary view of the nucleolus as a dynamic nuclear organelle capable of regulating numerous cellular processes has led to a re-evaluation of nucleolar protein function(s) (14).

The ARF tumor suppressor is localized to nucleoli in mammalian cells and plays an important role in preventing tumor development. Our initial studies have focused on identifying targets for ARF tumor suppression. One such target, NPM, was recently identified by our lab. Nucleophosmin (NPM/B23) is an abundant phosphoprotein localized in the granular regions of the nucleolus (22). NPM was found to be highly expressed in proliferating cells (7, 8), and has been associated with a variety of cellular phenomena, including ribosomal biogenesis, protein chaperoning and centrosome duplication (8, 13, 18, 19). Structurally, NPM can exist in both a monomeric and multimeric state, although NPM multimers seem to dominate in the nucleolus and may be crucial for the assembly of maturing ribosomes (16, 17, 24). More importantly, NPM, along with other nucleolar proteins, has been suggested to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may contribute to some of its essential functions (4). Indeed, NPM exit from the nucleolus/nucleus is an essential event in S phase progression; inhibition of this trafficking by the nucleolar tumor suppressor ARF results in cell cycle arrest (5). Additionally, NPM is an essential nucleolar protein with loss of its expression resulting in severe attenuation of cellular proliferation and increased apoptosis (3, 5, 9, 11), underscoring NPM's importance to the cell.

If nuclear exit of NPM plays a positive role in promoting cell growth and proliferation, what necessary function is it performing? While numerous proteins, such as Mdm2, cdc14p and TERT, are topologically restrained in the nucleolus following defined cellular cues, synthesis and export of newly synthesized ribosomal subunits from the nucleolus remains the only known nucleolar-specific event conserved throughout evolution (21). Recent work from *Xenopus laevis* and *Saccharomyces cerevisiae* has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (12) as well as a nuclear adaptor protein NMD3 that is conserved from yeast to man (23).

Despite the seemingly important role ARF plays in breast tumor prevention, with over half of all breast cancers lacking ARF expression, studying the interplay between ARF and its targets, like NPM, has remained a largely unexplored theme. In my original proposal, I aimed to use a variety of molecular and genetic methods to more accurately address the broad question of how ARF restrains breast cancer progression.

BODY

The ARF tumor suppressor is widely regarded as an upstream activator of p53-dependent growth arrest and apoptosis. However, recent findings indicate that ARF can also regulate the cell cycle in the absence of p53. In search of p53-independent ARF targets, we isolated nucleophosmin (NPM/B23), a protein we show is required for proliferation, as a novel ARF binding protein. In response to hyperproliferative signals, ARF is upregulated, resulting in the nucleolar retention of NPM and a concomitant cell cycle arrest. The Mdm2 oncogene out-competes NPM/B23 for ARF binding, and introduction of Mdm2 reverses ARF's p53-independent properties: in vitro NPM is released from ARF-containing protein complexes, and in vivo S phase progression ensues. ARF induction by oncogenes or replicative senescence does not alter NPM/B23 protein levels, but rather prevents its nucleocytoplasmic shuttling without inhibiting rRNA processing. By actively sequestering NPM in the nucleolus, ARF utilizes an additional mechanism of tumor suppression, one that is readily antagonized by Mdm2 (SEE ATTACHED PAPER #1 IN APPENDICES, "ARF Impedes NPM/B23 Shuttling in an Mdm2-Sensitive Tumor Suppressor Pathway").

In an effort to uncover the biological significance of NPM protein trafficking, we first discovered that NPM's exit from the nucleus also involved the classical CRM1-dependent nuclear export pathway. In search of targets for NPM export regulation, we discovered the ribosomal protein rpL5, a 60S subunit protein that chaperones the 5S ribosomal RNA into the nucleolus and out into the cytosol (15), was a direct binding partner for cytosolic and nuclear NPM proteins. We propose that NPM helps direct rpL5 nuclear export through a CRM1-dependent mechanism, providing NPM with direct access to the maturing ribosome and potential regulation of the translational machinery (SEE ATTACHED MANUSCRIPT #1 IN APPENDICES, "Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export").

Additionally we have discovered that NPM is a potent oncogene in the absence of the ARF tumor suppressor. NPM readily transforms fibroblasts without affecting centrosome number or genomic stability. We have also found NPM overexpressed in half of all breast carcinomas that we have evaluated (Figure 1).

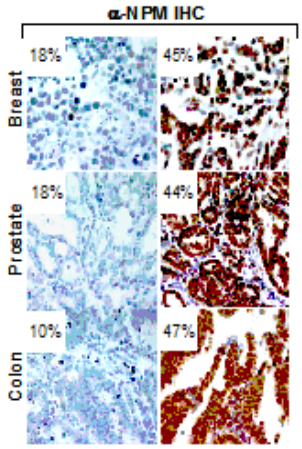

Figure 1. Primary human breast, prostate and colon carcinoma tissue microarrays were obtained and stained for NPM protein expression, with representative cancerous tissues staining negative for NPM shown on the left panels, and those staining extremely positive for NPM shown on the right panels. Statistics of NPM protein expression in all three carcinomas are indicated in the insets.

In addition to finding NPM overexpressed in nearly half of all breast carcinomas analyzed, we discovered that ARF was silenced or deleted in approximately 50% of all breast carcinomas. We will now go back and determine whether the loss of ARF function and overexpression of NPM are mutually exclusive genetic events in breast carcinomas or whether they can occur in the same tumor.

We have also begun work on generating a colony of transgenic mice overexpressing NPM in the breast epithelial compartment (through MMTV promoter). Currently, I have three founder mice that are positive for NPM overexpression in the breast epithelium.

KEY RESEARCH ACCOMPLISHMENTS

- ARF interacts with NPM *in vivo*
- NPM actively shuttles from the nucleolus to the cytoplasm
- ARF inhibits NPM shuttling
- NPM is a potent transforming oncogene
- NPM is overexpressed in ~50% of breast carcinomas
- *ARF* is silenced or deleted in ~50% of breast carcinomas

- "ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway", Brady et al. *Molecular and Cellular Biology* (2004) **21**:9327-9338.

- "Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export", Yu et al. Submitted (2005).

REPORTABLE OUTCOMES

- "ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway", Brady et al. *Molecular and Cellular Biology* (2004) **21**:9327-9338.
- "Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export", Yu et al. Submitted (2005).
- "The opposing roles of nucleophosmin and ARF" Oral Presentation at the annual Cold Spring Harbor Meeting on Oncogenes and Tumor Suppressors (August 2004)

- Received Ph.D. Degree in December 2004 from Washington University School of Medicine in St. Louis

- Experience in generating and breeding transgenic mice

CONCLUSIONS

This proposal was designed to investigate the opposing roles of ARF and NPM in the pathogenesis of breast cancer. In the first year of support, we have generated a significant amount of data that should help our lab and others understand the intricate mechanism(s) by which ARF targets NPM to suppress tumor formation. Additionally, we now know that NPM is overexpressed in human breast carcinomas and that in this context, it is a potent oncogene. We have published a paper on the significance of the ARF-NPM interaction and have submitted another manuscript that discusses a proposed role for NPM in promoting ribosome biogenesis. The latter could be quite important as it opens the door to a whole new array of putative anti-cancer targets that might be involved in protein translation. We have begun work on specific aim 2, having generated three founders that overexpress NPM in the breast epithelium. We are looking forward to another productive year working on this important project.

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APPENDICES

1. “ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway”, Brady et al. *Molecular and Cellular Biology* (2004) **21**:9327-9338.

2. “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yu et al. Submitted (2005).

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ARF Impedes NPM/B23 Shuttling in an Mdm2-Sensitive Tumor Suppressor Pathway

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The ARF tumor suppressor is widely regarded as an upstream activator of p53-dependent growth arrest and apoptosis. However, recent findings indicate that ARF can also regulate the cell cycle in the absence of p53. In search of p53-independent ARF targets, we isolated nucleophosmin (NPM/B23), a protein we show is required for proliferation, as a novel ARF binding protein. In response to hyperproliferative signals, ARF is upregulated, resulting in the nucleolar retention of NPM and concomitant cell cycle arrest. The Mdm2 oncogene outcompetes NPM/B23 for ARF binding, and introduction of Mdm2 reverses ARF's p53-independent properties: in vitro, NPM is released from ARF-containing protein complexes, and in vivo S phase progression ensues. ARF induction by oncogenes or replicative senescence does not alter NPM/B23 protein levels but rather prevents its nucleocytoplasmic shuttling without inhibiting rRNA processing. By actively sequestering NPM in the nucleolus, ARF utilizes an additional mechanism of tumor suppression, one that is readily antagonized by Mdm2.

The murine INK4a/ARF locus, encoding both the $p16^{INK4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in humans) tumor suppressors, exhibits an unparalleled efficiency of organization within a mammalian genome. Specifically, $p16^{INK4a}$ and $p19^{ARF}$ contain distinct promoters and first exons yet splice into a shared second exon that is translated in alternative reading frames (ARF) (33). While both proteins clearly contribute to tumor surveillance in mice and humans, they appear to play coordinate, yet independent, roles within the cell cycle. $p16^{INK4a}$ imposes a G_1/S phase block via direct inhibition of the cdk4 and cdk6 cyclin-dependent kinases, maintaining the active, hypophosphorylated state of the retinoblastoma (Rb) tumor suppressor (36). ARF, in response to hyperproliferative signals relayed by the expression of oncoproteins, such as Myc, E2F, E1A, and Ras, binds and sequesters Mdm2 in the nucleolus, thereby promoting p53-dependent pathways of growth arrest or apoptosis through stabilization of the nucleoplasmic pool of p53 (3, 9, 48). Additionally, ARF directly inhibits the ubiquitination of p53 by Mdm2, suggesting that nucleolar sequestration might not be a requisite step in ARF's activation of p53 (13, 46).

Mounting evidence suggests that the ARF-p53-Mdm2 pathway is not strictly linear. Mice engineered to overexpress a *Myc* transgene under the control of the immunoglobulin heavy chain enhancer (Eμ) develop B-cell lymphomas that exhibit biallelic *ARF* deletion, mutation of *p53*, or Mdm2 overexpression (11). Additional molecular analysis revealed that several tumors which lacked functional p53 also displayed Mdm2 overexpression, arguing against a simple epistatic relationship among *ARF*, *p53*, and *Mdm2* (11). Additionally, Carnero and colleagues showed that diminished ARF expression resulted in bypassing of replicative senescence, whereas induction of ARF restored ARF's tumor-suppressive properties, even in the presence of a dominant-negative p53 mutant (7). In support of these initial findings, two other groups have demonstrated the physiological significance of the p53-independent ARF pathway through examination of relevant mouse model systems. Mice lacking *ARF* primarily develop lymphomas and fibrosarcomas (18, 19); in contrast, *p53*-null animals consistently display lymphomas and osteosarcomas (16). Surprisingly, *ARF*/*p53* double-null mice exhibit multiple tumors with distinct origins, namely, the simultaneous presentation of carcinomas with lymphomas (45). In a corroborative study, wild-type and *p53*-null mice displayed normal development of the hyaloid vascular system in the eye, yet *ARF*-null and *ARF*/*p53* double-null animals failed to show proper regression of this structure (27). Collectively, these data reinforce the fact that ARF and p53 play shared, as well as unique roles, as sensors of inappropriate cell growth.

Clearly, genetic studies have confirmed the existence of an alternative, p53-independent ARF tumor surveillance pathway. In an effort to uncover novel players in this pathway, we isolated ARF-containing protein complexes from cells devoid of p53 and Mdm2. Here, we report the identification of nucleophosmin (NPM), a nucleolar phosphoprotein and clinical marker of highly proliferative cells, as a bona fide ARF-interacting protein. Recently, two studies have also described the formation of ARF-NPM complexes in mouse fibroblasts but drew two differing conclusions: ARF targets NPM/B23 for degradation (15) and/or ARF prevents rRNA processing (4). Our data offer a contrasting view. Specifically, ARF binds and retains NPM in the cell nucleolus, effectively impeding the nucleocytoplasmic shuttling of NPM, resulting in subsequent growth arrest. Importantly, Mdm2 antagonizes these effects, thereby preventing ARF-induced withdrawal from the cell cycle. Two intriguing implications of our findings are that (i)

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ARF, through its interaction with NPM, may directly access and inhibit additional cytoplasmic growth-promoting events and (ii) Mdm2, a well-established mediator of oncogenesis, dictates ARF's tumor-suppressive capacity in the absence of functional p53.

MATERIALS AND METHODS

Cell culture. Primary mouse embryonic fibroblasts (MEFs) (initially obtained from Gerard Zambetti, St. Jude Children's Research Hospital) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and 100 U of penicillin and streptomycin (GIBCO/BRL, Gaithersburg, Md.). Virus production and infection of primary MEFs were carried out according to methods described previously, using retroviral helper and vector plasmids (34) provided by Charles Sawyers (University of California—Los Angeles).

Plasmid constructs. Vectors encoding $p19^{ARF}$, $p19^{ARF}$ Δ1-14, and Mdm2 (210-304) were used as previously described (44) or were subcloned into the EcoRI sites of pEGFP-C1 plasmids (Clontech). A retroviral plasmid encoding full-length Mdm2 was a generous gift from Martine Roussel (St. Jude's Children's Research Hospital). Full-length NPM was cloned from wild-type MEF reverse-transcribed RNAs using the following PCR primers: 5′-GCGCATATG GAAGACTCGATGGATATGGAC-3′ (sense) and 5′-GCGGGATCCTTAAA GAGATTTCCTCCACTGCCAGAG-3′ (antisense). The NPM PCR product was digested with the NdeI and BamHI restriction enzymes and subcloned into the NdeI and BamHI sites of the pET28a vector (Novagen, Madison, Wis.). The pET28a six-histidine-tagged NPM insert was PCR subcloned using the following primers: 5′-GCGGAATTCATGGGCAGCAGCCATCATCAT-3′ (sense) and 5′-GCGGAATTCTTAAAGAGATTTCCTCCACTG-3′ (antisense). The resultant PCR product was subcloned into the EcoRI site of the pSRα-MSV-tkneo retroviral vector. A retroviral vector encoding DMP1 was a generous gift from Charles J. Sherr (St. Jude's Children's Research Hospital).

Nucleolar isolation and MALDI-TOF. TKO MEFs labeled with [^{35}S]methionine and infected for 96 h with retroviruses encoding hemagglutinin-tagged ARF (HA-ARF) were harvested in ice-cold phosphate-buffered saline (PBS) and homogenized with a glass douncer. Nucleoli were isolated as previously described (2). Nucleoli were verified by staining them in 0.1% azure C dye (Sigma, St. Louis, Mo.). The purified nucleoli were lysed in 25 mM Tris-HCl, pH 7.4–150 mM NaCl–1 mM $MgCl_2$–1 mM EDTA–1% Tween-20 and sonicated with three 10-s bursts at 20% output. HA-ARF complexes were immunoprecipitated with antibodies against the HA epitope conjugated to protein A-Sepharose (Amersham, Piscataway, N.J.). Proteins were eluted with 100 mM glycine (pH 3.0), neutralized in 1 M Tris-HCl (pH 7.4), and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to polyvinylidine difluoride (PVDF) membranes (Millipore, Bedford, Mass.) and exposed to film. Identified bands were excised and digested with 0.1 μg of trypsin per ml (Promega, Rockford, Ill.) overnight at 30°C. Tryptic peptide fragments were extracted with 60% acetonitrile and 0.1% trifluoroacetic acid using an automated Multiprobe II system (Packard Biosciences, Meriden, Conn.). The extracted peptides were dried under vacuum, purified with Zip Plates (Millipore), resuspended in matrix, and subjected to matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis using a Voyager DE Pro spectrometer (Applied Biosystems, Foster City, Calif.).

Immunoprecipitation and Western blot analysis. Wild-type, DKO (*p53*/*Mdm2* null), and TKO (*ARF*/*p53*/*Mdm2* null) MEFs were infected with retroviruses encoding MycER (a gift from J. Bishop), DMP1, ARF, or ARF Δ1-14 and lysed in binding buffer (25 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 0.4 U of aprotinin) either 48 (wild type and DKO) or 96 (TKO) h after infection. For MycER infections, cells were lysed 48 h after the addition of 4-hydroxytamoxifen (4-HT) (1 μM). Antibody to the ARF C terminus (a gift from Charles Sherr), antibody to NPM (Zymed, San Francisco, Calif.), or nonimmune rabbit serum (NRS) or nonimmune mouse serum was added to the binding reaction mixtures for 1 h at 4°C. Immune complexes were precipitated with protein A-Sepharose and washed with binding buffer. The precipitated proteins, as well as direct protein lysates, were separated by SDS-PAGE and transferred to PVDF membranes preactivated in methanol. Mdm2, ARF, NPM, and γ-tubulin proteins were visualized by direct immunoblotting with 2A10, ab80 (abcam, Cambridge, United Kingdom), NPM (Zymed), and γ-tubulin (Santa Cruz, Santa Cruz, Calif.) antibodies, respectively.

Immunofluorescence and confocal microscopy. Wild-type or TKO MEFs (3×10^4) were seeded onto glass coverslips and infected with retroviruses encoding MycER, tkNeo (vector), ARF, or ARF in combination with Mdm2 or NPM. The cells were fixed 96 h after infection (TKO MEFs) or 48 h after the addition of 4-hydroxytamoxifen (MycER-infected wild-type MEFs) with ice-cold methanol-acetone (1:1 [vol/vol]) and stained for 1 h with either a rabbit ARF C-terminal antibody (4 μg per ml), human fibrillarin (Sigma), or mouse NPM antibody (4 μg per ml), followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rabbit immunoglobulin (Pierce) or tetramethyl rhodamine isothiocyanate-conjugated anti-rabbit or anti-human immunoglobulin (Pierce). For measurement of DNA replication, 5-bromodeoxyuridine (BrdU) (Sigma) was added to the culture medium 72 h after infection at a final concentration of 10 μM. Twenty-four hours after the addition of BrdU, the cells were fixed in ice-cold methanol-acetone as described above, treated for 10 min with 1.5 N HCl, and stained for 1 h with a mouse monoclonal anti-BrdU antibody (Amersham), followed by FITC-conjugated anti-mouse immunoglobulin (Pierce). Nuclei were visualized with DAPI (4′,6-diamidino-2-phenylindole) (Sigma). At least 100 cells were counted on each of three coverslips for all experimental conditions. Fluorescence signals were detected using a Nikon epifluorescent compound microscope (100×) fitted with a Nikon FDX-35 charge-coupled device camera.

FPLC. ARF synthetic peptides were coupled to cyanogen bromide-activated Sepharose (Amersham) and equilibrated as previously described (44). TKO lysates (200 μg) were injected at a flow rate of 1.0 ml per min, washed with 20 ml of 25 mM Tris-HCl (pH 7.4) at 1.0 ml per min, and eluted with a 25 ml of NaCl gradient (0 to 1.5 M) at 1.0 ml per min, followed by 20 ml of 100 mM glycine (pH 3.0) at 1.0 ml per min, using BioLogic fluid phase liquid affinity chromatography (FPLC) and BioLogic HR software (Bio-Rad, Hercules, Calif.). The 1.0-ml collected fractions were precipitated with trichloroacetic acid, resuspended in 1 M Tris-HCl (pH 8.0), and electrophoretically separated on denaturing polyacrylamide gels containing SDS. The separated proteins were transferred to PVDF membranes, and individual proteins were detected using antibodies to cyclin A (Santa Cruz), fibrillarin (Sigma), NPM (Zymed), and Mdm2 (2A10).

Heterokaryon assay. HeLa cells (2×10^5) were seeded onto glass coverslips in six-well plates and transfected with His-NPM alone or in combination with green fluorescent protein (GFP)-ARF, GFP-ARF N62, or GFP-ARF Δ1-14. As a positive shuttling control, Myc-tagged NPC-M9 (a gift from J. Alan Diehl, University of Pennsylvania) was transfected in combination with GFP-ARF N62. NIH 3T3 cells (6×10^5) were seeded onto the HeLa cells 24 h posttransfection and cocultured for an additional 16 h at 37°C. The cocultures were then incubated for 30 min with DMEM containing cycloheximide (100 μg per ml). For fusion of mouse and human plasma membranes, the medium was removed and the cells were incubated with 50% polyethylene glycol in PBS (prewarmed to 37°C) for 105 s, followed by three PBS washes (prewarmed to 37°C). The cocultures were incubated with DMEM containing cycloheximide (100 μg per ml) for an additional 4 h to permit protein shuttling. Heterokaryons were fixed with formalin-methanol (10% [vol/vol] in H_2O) for 15 min and permeabilized with 1% NP-40 in PBS for 5 min at room temperature, followed by three PBS washes. The cells were then blocked for 1 h with 5% fetal bovine serum in PBS and stained for 1 h with a rabbit anti-His antibody (1:25; Santa Cruz) or mouse anti-myc antibody (1:10; Santa Cruz), followed by either FITC-conjugated anti-rabbit or -mouse immunoglobulin (Pierce) or rhodamine X-conjugated anti-rabbit or -mouse immunoglobulin (Pierce) for 30 min. Nuclei were stained with DAPI. Fluorescent signals were detected using a Nikon epifluorescent compound microscope (100×) fitted with a Nikon FDX-35 charge-coupled device camera.

rRNA processing. HeLa cells (10^6) were seeded onto 60-mm-diameter dishes and transfected with plasmids encoding GFP or GFP-ARF. The cells were pulse-labeled for 45 min with [5,6-^{3}H]uridine (Amersham) and chased with complete medium for 1 h as previously described (39). Total RNA was harvested from cells using Trizol (GIBCO), and labeled rRNAs (2,000 cpm/lane) were separated on 1% formaldehyde-agarose gels. The gels were denatured for 20 min (0.05 N NaOH, 0.15 M NaCl) and neutralized for 30 min (0.1 M Tris, pH 7.5, 0.15 M NaCl) before being transferred to Hybond N+ membranes. Labeled rRNAs were visualized on EN^{3}HANCE (Perkin-Elmer)-sprayed membranes using standard autoradiography.

RNA interference. The following 19-nucleotide duplex, corresponding to nucleotides 133 to 151 downstream of the murine NPM transcriptional start site, was synthesized and cloned into the pSUPER.retro plasmid vector (Oligo-Engine, Seattle, Wash.) according to the manufacturer's instructions: 5′-AGAA CGGTCAGTTTAGGAG-3′ (sense) and 5′-CTCCTAAACTGACCGTTCT-3′ (antisense). Retroviruses encoding NPM-targeting small interfering RNAs (siRNAs) were generated via cotransfection of pSUPER.retro-NPM RNA interference (RNAi) and helper plasmids into packaging cells as described above. TKO MEFs seeded onto 100-mm-diameter dishes were infected with pSUPER-NPM RNAi retroviruses and subjected to Western blotting and indirect immunofluorescent analyses 96 h after retroviral transduction, using antibodies de-

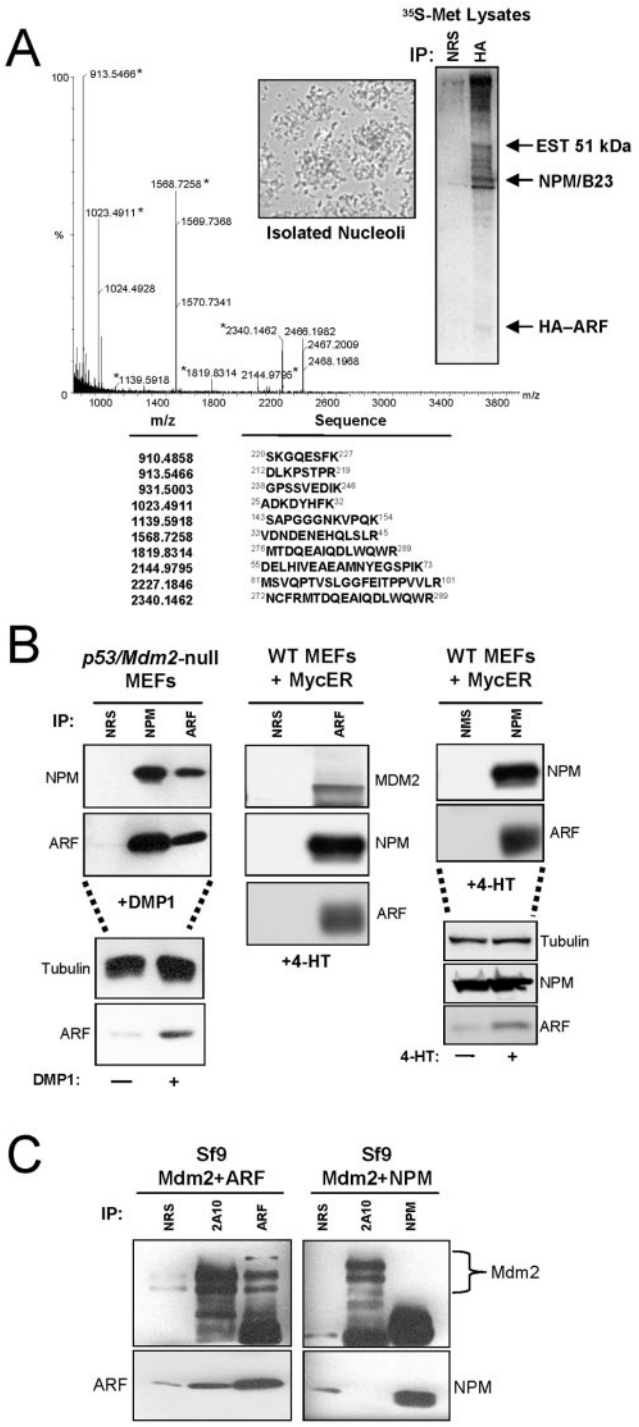

FIG. 1. NPM interacts with ARF in response to hyperproliferative signals. (A) TKO (*p53*/*ARF*/*Mdm2* null) MEFs infected with retroviruses encoding HA-tagged ARF in the presence of [^{35}S]methionine were harvested in cold PBS. Nucleoli were isolated from TKO extracts (inset), and purified nucleolar proteins were immunoprecipitated (IP) with nonimmune rabbit serum or antibodies recognizing the HA epitope. The indicated radiolabeled protein band at ~38 kDa was excised, digested with trypsin, and resuspended in matrices. The sample was subjected to MALDI-TOF analysis, and resultant tryptic peptide molecular masses were entered into the Prospector Database search engine for protein identification. (B) DKO (*p53*/*Mdm2*-null) and wild-type (WT) MEFs infected with retroviruses encoding DMP1 or tamoxifen-inducible MycER were harvested and lysed 48 h after infection (DMP1) or induction (MycER) with 4-HT. NPM and ARF proteins were immunoprecipitated with NRS, normal mouse serum (NMS), monoclonal antibody to NPM, or a rabbit polyclonal antibody directed to the ARF C terminus (ARF). Precipitated proteins were electrophoretically separated on denaturing gels, transferred to PVDF membranes, and immunoblotted with the indicated antibodies. Protein induction and loading controls are shown below each IP for each condition. (C) Sf9 insect cells infected for 48 h with baculoviruses encoding Mdm2 and ARF or Mdm2 and NPM were harvested and lysed. Mdm2, ARF, and NPM proteins were immunoprecipitated with NRS, monoclonal antibody to Mdm2 (2A10), monoclonal antibody to NPM, or a polyclonal antibody directed to the ARF C terminus (ARF). Precipitated proteins were electrophoretically separated on denaturing gels, transferred to PVDF membranes, and immunoblotted with the same antibodies.

scribed above. For indirect immunofluorescent analysis of NPM expression and BrdU incorporation, cells infected on 100-mm-diameter dishes were seeded onto glass coverslips (10^5 per coverslip) 24 h after infection, followed by addition of BrdU (10 μM), fixation, and staining for NPM and BrdU at 96 h posttransduction, as described above. Nuclei were demarcated with DAPI, and fluorescence was visualized as described above. As a control, TKO MEFs were infected with empty siRNA vector and scrambled siRNAs (gifts from Helen Piwnica-Worms) (5′-AGGGATGTGTCCCCTTGTG-3′ [sense] and 5′-CTCTTGGGGTCTCTTCCC-3′ [antisense]) and were assayed in parallel with those infected with pSUPER-NPM RNAi retroviruses.

RESULTS

MALDI-TOF analysis reveals that NPM is a component of the ARF complex in vivo. ARF antagonizes the numerous functions of Mdm2, including the ubiquitination (13, 28, 46) and nuclear export of p53 (41), via direct binding of two conserved domains within ARF to two central regions within Mdm2 (6, 10, 26, 44). Through this interaction, ARF mobilizes Mdm2 into the cell nucleolus, permitting nucleoplasmic accumulation of active p53 molecules (25, 26, 41, 43). However, in limited settings, nucleolar sequestration of Mdm2 appears not to be required for ARF's activation of p53 (21, 24).

To establish the biochemical composition of an Mdm2-free ARF complex, we infected MEFs derived from mice lacking *ARF*, *p53*, and *Mdm2* (TKO) with retroviruses encoding HA-ARF and labeled cellular proteins with [^{35}S]methionine. To reduce nonspecific binding of abundant cytoplasmic and nuclear proteins during cell lysis, HA-ARF complexes were isolated from purified nucleoli. Intact nucleoli (Fig. 1A, inset) were lysed, and ARF-containing protein complexes were precipitated using antibodies against the HA moiety (Fig. 1A). HA-ARF is difficult to detect at ~20 kDa due to its extreme lack of methionine and cysteine residues. Proteins within ARF complexes were separated via SDS-PAGE, digested with trypsin to obtain individual peptide fragments, and combined with matrices. Dried matrix-peptide mixtures were subjected to MALDI-TOF analysis. Peptide masses obtained from a protein band at ~38 kDa matched the peptide fingerprint for murine NPM (Fig. 1A). An abundant nucleolar phosphoprotein, NPM is upregulated in response to mitogenic signals (12, 20) and is necessary for entry into S phase of the cell cycle in established fibroblast cell lines (17). NPM's reported role as a marker of cell proliferation prompted us to further investigate its association with the ARF tumor suppressor.

We next examined the ARF-NPM interaction in a physio-

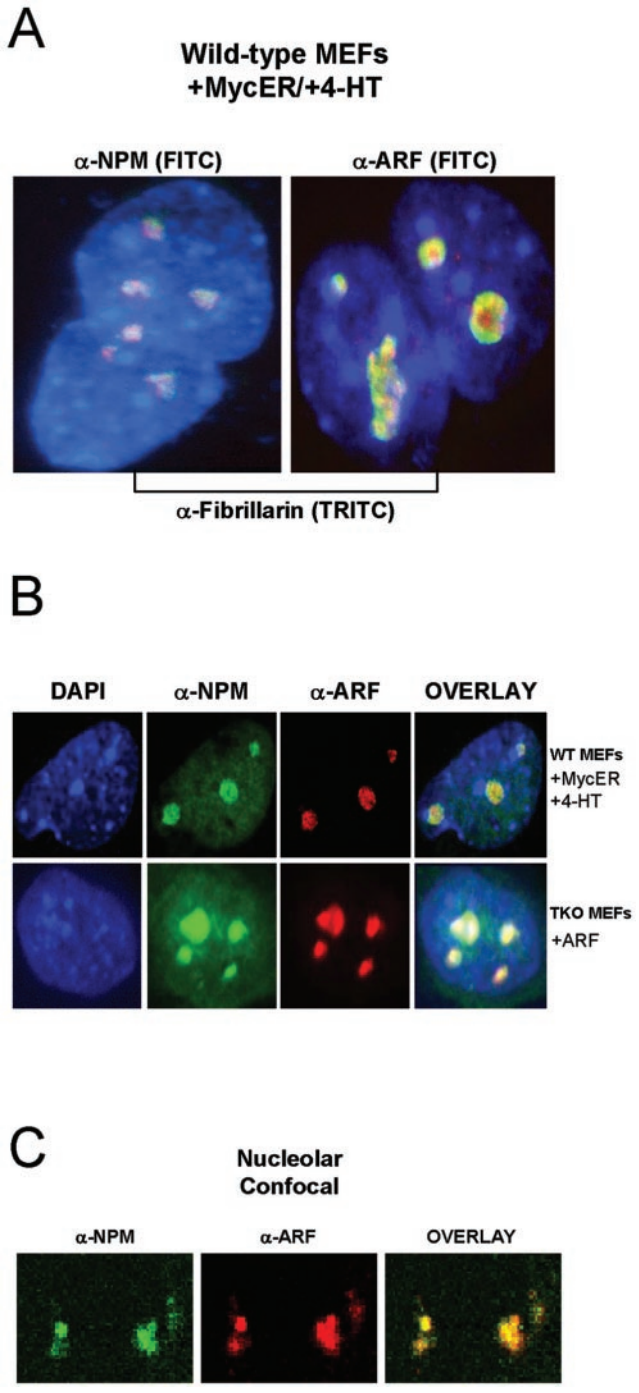

FIG. 2. ARF colocalizes with NPM in the granular region of the nucleolus. (A) Wild-type (WT) MEFs were fixed and immunostained for NPM, fibrillarin, and ARF to mark nucleolar structures. WT MEFs infected with MycER were treated with 4-HT for 48 h to induce ARF expression. (B) WT MEFS infected with MycER retroviruses and treated with 4-HT were fixed and stained with antibodies against ARF (red) and NPM (green) 48 h after 4-HT addition. TKO MEFs infected with retroviruses encoding ARF were fixed and stained using antibodies against NPM (green) and the ARF C terminus (red) 96 h after infection. Nuclei were demarcated with DAPI staining. All immunofluorescent microscopy images in (A) and (B) were captured at ×100 magnification. (C) Confocal microscopy was performed on the same ARF-infected TKO MEFs shown in (B). Overlap of staining was verified using Adobe Photoshop (yellow). The staining pattern shown was exhibited by >95% of cells viewed in four independent experiments.

logically relevant in vivo setting, in which hyperproliferative signals were used to induce endogenous ARF expression in MEFs possessing an intact *ARF* locus. Specifically, MEFs derived from *p53*/*Mdm2*-null mice (DKO) were infected with retroviruses encoding the DMP1 transcription factor, a direct activator of the ARF promoter in response to hyperproliferative signals such as myc (14). DMP1 transduction resulted in the upregulation of ARF protein levels, and subsequent coimmunoprecipitation experiments, using antibodies raised against ARF or NPM, demonstrated the in vivo formation of the ARF-NPM protein complex (Fig. 1B, left). Similarly, wild-type MEFs infected with retroviruses encoding a tamoxifen-inducible form of the myc oncoprotein (MycER) displayed increased ARF protein levels upon treatment with 4-HT, and coimmunoprecipitation experiments again verified the ARF-NPM interaction (Fig. 1B, middle and right). As expected, induction of ARF in wild-type MEFs also resulted in the coimmunoprecipitation of Mdm2 with ARF (Fig. 1B, middle). To ensure that Mdm2 was not mediating the ARF-NPM interaction in wild-type MEFs via potential formation of ternary complexes, we investigated whether NPM could bind directly to Mdm2. Whereas ARF readily complexed with Mdm2, NPM failed to bind to Mdm2 (Fig. 1C), demonstrating that the ARF-NPM and ARF-Mdm2 interactions represent distinct and independent protein complexes.

ARF colocalizes with NPM in the granular region of the nucleolus. In mouse and primary diploid human cells, ARF is expressed at low basal levels and localizes to the granular region of the nucleolus (21, 25, 32, 43), a subnuclear organelle that is readily detected upon immunostaining for fibrillarin. NPM, a highly abundant nucleolar protein, localizes primarily to fibrillarin-positive nucleoli, but additional staining throughout the nucleus is observed (Fig. 2A and C). To corroborate our finding that ARF and NPM interact and reside within the same protein complex, we next wanted to verify that NPM colocalizes with ARF in the granular region of the nucleolus under conditions of ARF induction in vivo. As expected, myc-mediated upregulation of endogenous ARF in wild-type MEFs resulted in the accumulation of ARF in nucleoli, as indicated by the colocalization of ARF with fibrillarin (Fig. 2A). Similarly, retroviral transduction of exogenous ARF into TKO MEFs produced a robust nucleolar pattern of ARF expression (Fig. 2B). Coimmunostaining for NPM and ARF in myc-transduced wild-type MEFs and ARF-transduced TKO MEFs revealed that NPM and ARF colocalize to the granular region of nucleoli, as confirmed by indirect immunofluorescent (Fig. 2B, overlay) and confocal (Fig. 2C, overlay) microscopy.

ARF utilizes its extreme amino terminus in binding NPM. The first 14 amino-terminal residues of murine ARF (ARF 1 to 14) represent the most highly conserved domain among all ARF orthologs and are indispensable for ARF's p53-dependent and -independent properties (44, 45). To test our hypothesis that NPM interacts with these functionally conserved residues within ARF, we investigated whether an amino-terminal deletion mutant lacking ARF's first 14 residues (ARF Δ1-14) could interact with NPM in vivo. TKO MEFs were infected with retroviruses encoding full-length ARF or ARF Δ1-14, followed by coimmunoprecipitation against ARF and NPM to assess formation of the ARF-NPM complex. Whereas full-length murine ARF readily bound NPM (Fig. 3A, upper left)

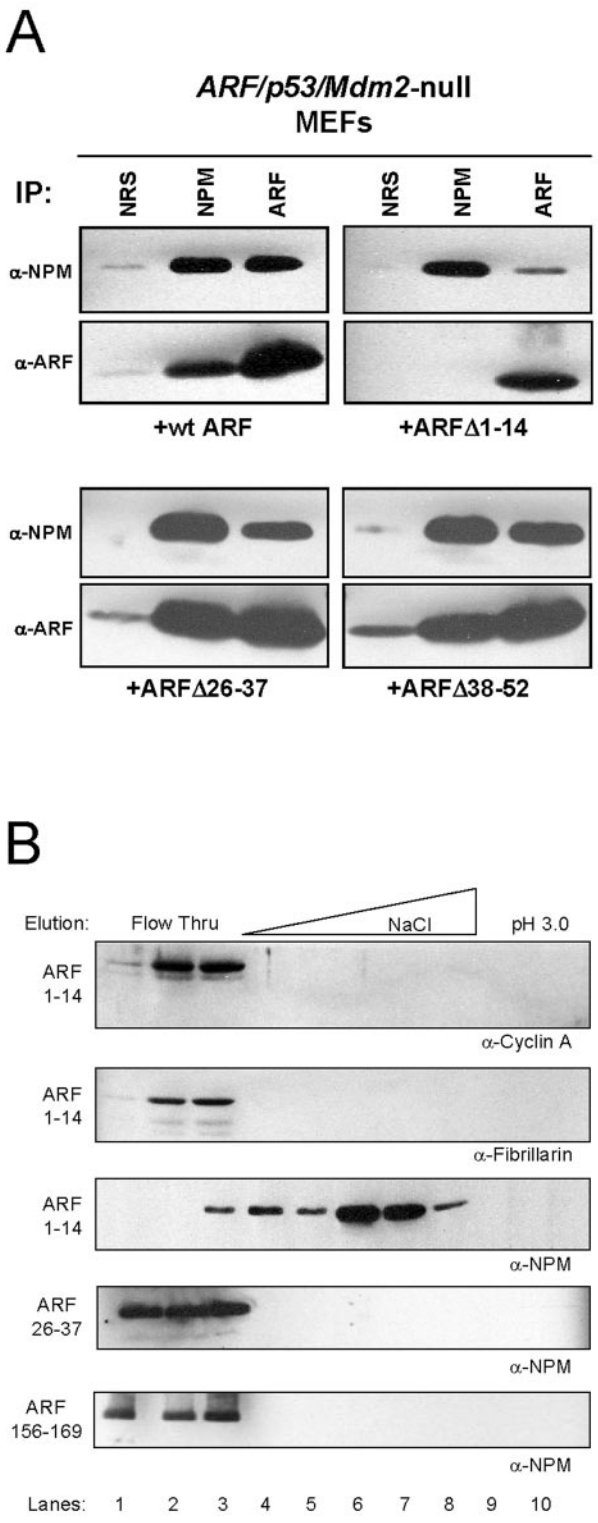

FIG. 3. NPM binds to the amino terminus of ARF. (A) TKO MEFs infected with ARF, ARF Δ1-14, ARF Δ26-37, or ARF Δ38-52 were harvested and lysed 96 h after infection. NPM and ARF proteins were immunoprecipitated (IP) with NRS, monoclonal antibody to NPM, or a polyclonal antibody directed to the ARF C terminus (ARF). Precipitated proteins were electrophoretically separated on denaturing gels, transferred to PVDF membranes, and immunoblotted with the same antibodies. (B) TKO lysates were injected onto the indicated ARF peptide FPLC columns, washed (lanes 1 to 3), and eluted with an NaCl gradient (lanes 4 to 8), followed by acid (lanes 9 and 10) as shown. Trichloroacetic acid-precipitated proteins were separated on denaturing polyacrylamide gels and immunoblotted with antibodies against cyclin A, fibrillarin, and NPM.

ARF Δ1-14 failed to exhibit a significant in vivo interaction with NPM (Fig. 3A, upper right), consistent with its inability to induce complete cell cycle arrest in TKO MEFs (45). However, we did observe some residual binding of this mutant (Δ1-14) with NPM, leaving open the possibility that other regions within ARF may be important in mediating ARF-NPM interactions in vivo. Contiguous deletion of ARF residues 26 to 37 (which define the low-affinity Mdm2 binding site) or 38 to 52 did not alter ARF-NPM complex formation (Fig. 3A, bottom), and unlike ARF Δ1-14, these mutants are fully capable of inducing cell cycle arrest (43).

Interestingly, ARF's amino terminus, which contains several conserved arginine residues, exhibits a local charge (pI = 10.8) comparable to that of the full-length ARF protein (pI = 11.2), suggesting that ARF, in the absence of its amino terminus, could utilize its other positively charged regions to mediate critical protein-protein interactions. However, previous studies of the ARF-Mdm2 complex have demonstrated that ARF's selection of physiologically relevant targets is based on more than simple electrostatic affinity (6, 10). To define the specificity of NPM's interaction with the ARF amino terminus, we injected whole-cell lysates from TKO MEFs onto affinity columns comprised of similarly charged ARF peptides and eluted bound proteins. Cyclins (A, B, D, and E) and their respective catalytic cdk partners (2, 1, 4/6, and 2, respectively) do not specifically bind to ARF (J. D. Weber, unpublished observation), and elution profiles from ARF peptide affinity columns confirmed this (Fig. 3B, lanes 1 to 3). NPM bound to the first 14 residues of ARF and eluted in the salt gradient (Fig. 3B, lanes 4 to 8), whereas other abundant and equally acidic nucleolar proteins, such as fibrillarin, did not (Fig. 3B, lanes 1 to 3). Importantly, NPM did not interact with ARF residues 26 to 37, the low-affinity site for Mdm2 association, and also failed to bind amino acids 156 to 169 of ARF, a region with a basic charge content equal to that of residues 1 to 14 (Fig. 3B).

Mdm2 releases NPM from ARF-containing protein complexes. Previous work established ARF's reasonably strong affinity for a central region of Mdm2 (residues 210 to 304) (6, 10), and interestingly, both Mdm2 and NPM interact with the first 14 amino acids of ARF (Fig. 3B) (44). To compare the relative binding strength of the ARF-NPM association with that of ARF-Mdm2, we injected purified full-length NPM onto a peptide column composed of ARF residues 1 to 14. NPM did not interact with the Sepharose used to make the column (Fig. 4A, top, lanes 1 to 3) and was not nonspecifically outcompeted once it was bound to the ARF 1 to 14 peptide column (Fig. 4A, bottom, lanes 4 to 8). Once NPM had bound to the ARF column (Fig. 4B, diagram), the column was washed with buffer containing an excess molar amount of purified Mdm2 residues 210 to 304, which corresponds to the ARF binding domain (6, 44). Bound proteins were eluted with a salt gradient followed by an acid wash, and the resultant fractions were subjected to Western blot analysis for detection of NPM and Mdm2. Ini-

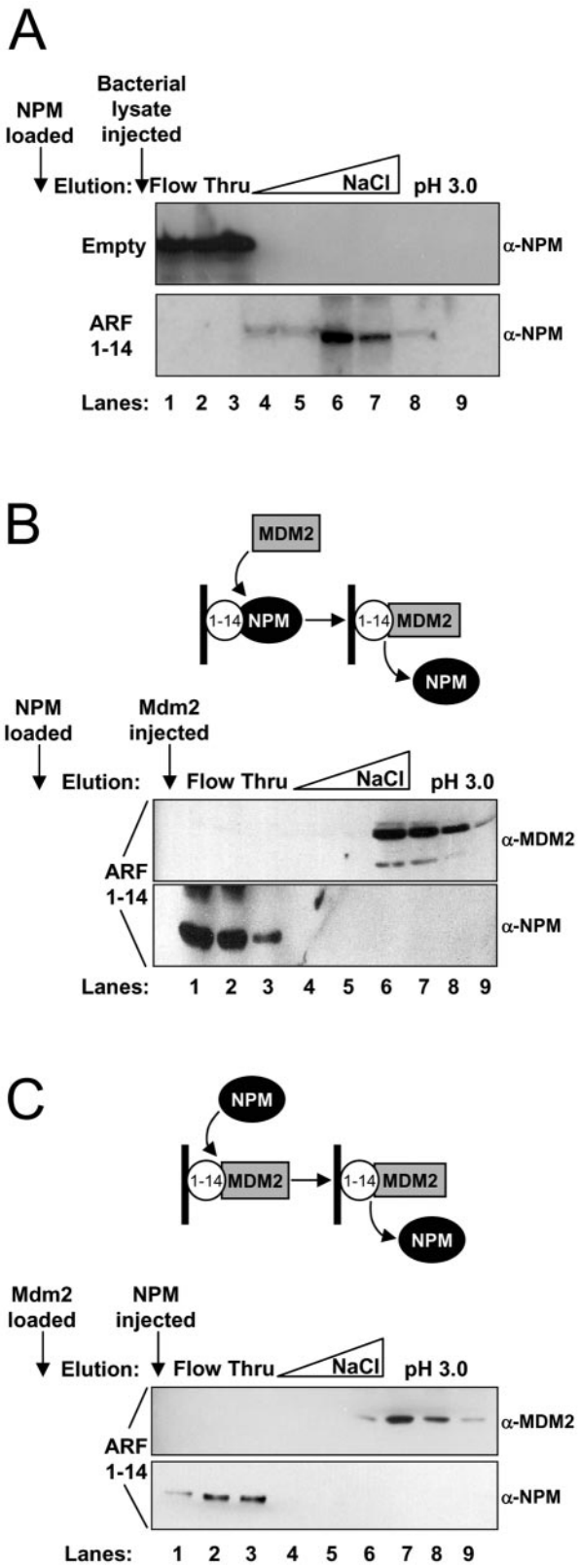

FIG. 4. NPM and Mdm2 compete for the conserved ARF amino terminus. Affinity-purified (A and B) NPM (1 μM) or (C) Mdm2 (1 μM) containing residues 210 to 304 was initially loaded onto either an empty or ARF 1 to 14 peptide affinity column and washed. Subsequently, (A) nonspecific bacterial lysate, purified recombinant (B) Mdm2 (1 μM), or (C) NPM (1 μM) was injected onto the ARF 1 to 14 column (arrows), washed (lanes 1 to 3), and eluted with an NaCl gradient (0.5 to 1.5 M) (lanes 4 to 6), followed by acid (pH 3.0) (lanes 7 to 9). Fractions from the flowthrough and elution steps were collected, separated on denaturing polyacrylamide gels, and immunoblotted with antibodies against NPM and Mdm2 (H221).

tially, NPM was retained on the ARF column yet was exchanged for Mdm2 in subsequent wash steps and eluted in the flowthrough (Fig. 4B, lanes 1 to 3). Newly formed ARF-Mdm2 complexes were stable but eventually eluted under high-salt or acidic conditions (Fig. 4B, lanes 6 to 9), demonstrating ARF's preference for Mdm2 over NPM as a binding partner. In agreement with this finding, Mdm2 remained bound to the ARF peptide column when the column was washed with buffer containing an excess molar amount of purified full-length NPM (Fig. 4C, lanes 6 to 9).

Mdm2 antagonizes ARF's p53-dependent and -independent functions. ARF induction evokes a G_1 phase cell cycle arrest in wild-type and TKO MEFs via p53-dependent and -independent mechanisms, respectively (45). Importantly, both lines of MEFs failed to arrest when Mdm2 was transduced in concert with ARF (Fig. 5A), presumably due to Mdm2's capacity to (i) terminate the p53 response in wild-type cells and (ii) release NPM from ARF complexes in TKO MEFs via preferential binding of Mdm2 to nucleolar ARF (Fig. 4B and C). Consistent with these findings, introduction of exogenous NPM into ARF-transduced TKO MEFs significantly rescued the cells' capacity to proceed through G_1 and into S phase (Fig. 5B). However, overexpression of NPM in ARF-transduced wild-type MEFs was not sufficient to override ARF's p53-dependent cell cycle inhibition (Fig. 5B). Notably, this could be attributed to ARF's ability to induce the p53 effector, $p21^{CIP1}$, in wild-type MEFs but not in TKOs (45, 48). These findings suggest that restoration of Mdm2 expression in ARF-transduced TKO MEFs triggers a switch in ARF's binding partners, prompting NPM's release from ARF-containing protein complexes.

Given that overexpression of NPM was sufficient to bypass ARF-induced growth arrest in TKO MEFs but not wild-type MEFs (Fig. 5B), we wanted to verify that ARF's cell cycle arrest was not mediated through simple downregulation of NPM protein expression. In contrast to a recent report (15), NPM protein levels were not reduced in response to ARF transduction in TKO MEFs (Fig. 5C, right). A hallmark of mouse cell culture-induced cellular senescence is the gradual induction of ARF (48). ARF accumulation leads to cell cycle arrest, and appropriately, mouse fibroblasts lacking *ARF* are immortal and can be passaged indefinitely (18). To test whether ARF accumulation lowered NPM protein levels in this physiological setting, we passaged wild-type MEFs on a 3T3 protocol (18) and assayed for ARF and NPM protein expression. As cells accumulated ARF protein, protein levels of NPM failed to decline and remained steady throughout fibroblast passaging, demonstrating that ARF does not reduce NPM protein levels to arrest cell growth (Fig. 5C, left). This finding, in combination with others' observations that NPM shuttles rapidly into the nucleus (5, 47), led us to hypothesize that nucleolar ARF might inhibit NPM's transit throughout the cell, effectively negating NPM's growth-promoting poten-

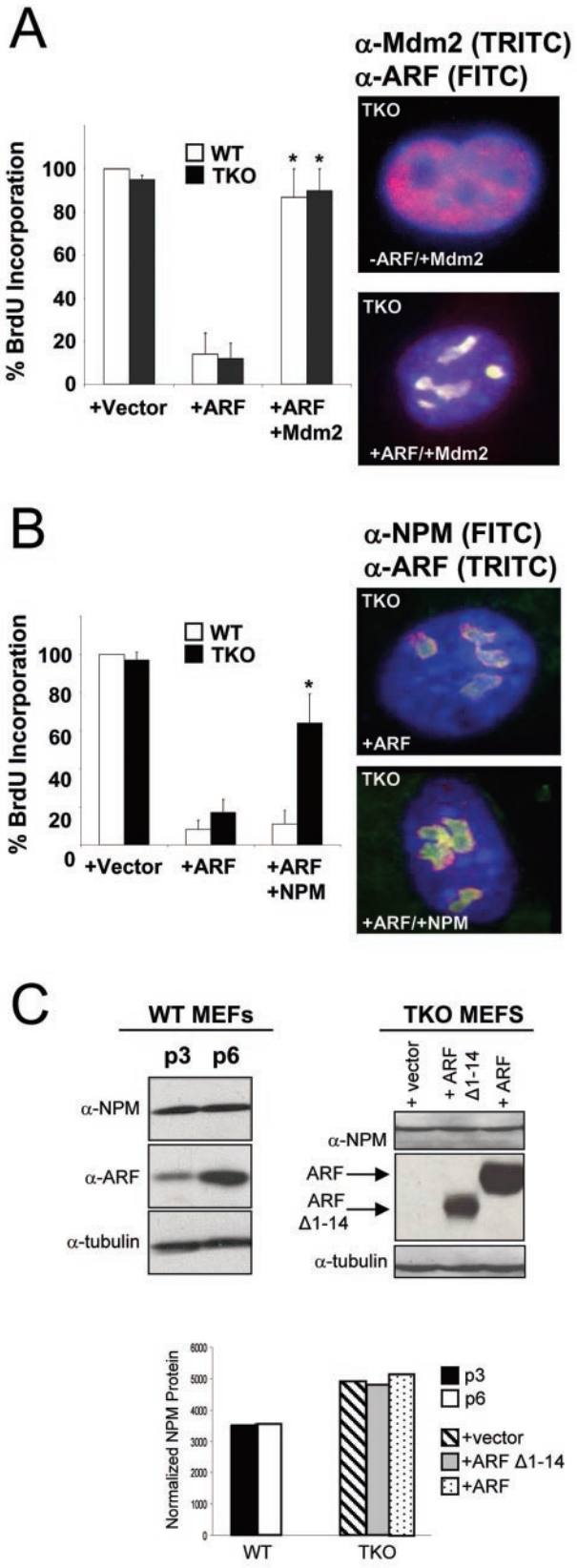

FIG. 5. Overexpression of Mdm2 or NPM antagonizes ARF-induced cell cycle arrest. (A) Primary wild-type (WT) and TKO MEFs were infected with retroviruses encoding tk Neo (vector), ARF, or ARF in combination with Mdm2. The cells were labeled with BrdU 24 h prior to fixation (48 h for WT; 96 h for TKO [postinfection]). The cells were stained with antibodies recognizing incorporated BrdU. The error bars indicate standard deviations of at least 100 cells in three independent experiments (*, $P > 0.005$). TKO MEFs infected with ARF in combination with Mdm2 were fixed 96 h postinfection and stained with antibodies recognizing Mdm2 (red) and ARF (green); regions of yellow indicate overlap of Mdm2 and ARF. Nuclei were stained with DAPI (blue). (B) Primary wild-type (WT) and TKO MEFs were infected with retroviruses encoding tkNeo (vector), ARF, or ARF in combination with NPM. The cells were labeled with BrdU 24 h prior to fixation (48 h for WT; 96 h for TKO [postinfection]). The cells were stained with antibodies recognizing incorporated BrdU. The error bars indicate standard deviations of 100 cells counted in three independent experiments (*, $P > 0.005$). TKO MEFs infected with ARF in combination with NPM were fixed 96 h postinfection and stained with antibodies recognizing NPM (green) and ARF (red); regions of yellow indicate overlap of NPM and ARF. Nuclei were stained with DAPI (blue). (C) Wild-type MEFs were passaged on a 3T3 protocol and collected at passages 3 and 6. TKO MEFs infected with retroviruses encoding tkNeo (vector), ARF Δ1-14, or ARF were harvested and lysed 96 h postinfection. Whole-cell lysates were separated on denaturing polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM, ARF, and γ-tubulin (loading control). NPM protein was normalized to γ-tubulin using ImageJ software and graphed as arbitrary normalized units.

tial via topological restraint rather than protein degradation or turnover.

ARF prevents NPM's nucleocytoplasmic shuttling. As an upstream activator of p53-dependent pathways of growth arrest and apoptosis, ARF stabilizes p53 protein levels through binding and sequestration of Mdm2 in the nucleolus, away from nucleoplasmic pools of p53, suggesting that regulation of protein topology may be an underlying feature of ARF-mediated tumor suppression (25, 41, 43). The notion that subcellular compartmentalization can either enable or disable a protein's function is further supported by our present understanding of NPM dynamics within the cell. Previous studies have established NPM's participation in nucleocytoplasmic shuttling (5, 47), while more recent work indicates that a relatively small fraction of NPM appears to transit from the nucleolus to the cytosol in a cell cycle-dependent manner (29, 42). Given that nucleolar ARF limits Mdm2's oncogenic potential by preventing its access to, and export from, the nucleus (41, 43), we hypothesized that ARF may utilize a similar strategy to inhibit NPM function, thereby achieving p53-independent command of the cell cycle.

To address ARF's potential role in regulating NPM trafficking throughout the cell, we conducted in vivo heterokaryon shuttling assays (41). An expression construct encoding six-histidine-tagged full-length murine NPM (His-NPM) was transiently transfected alone or in combination with green fluorescent protein-tagged constructs encoding either full-length murine ARF (GFP-ARF) or ARF deletion mutants that either retain (GFP-ARF N62) or lack (GFP-ARF Δ1-14) the conserved amino terminus into asynchronously growing HeLa cells. Mouse NIH 3T3 fibroblasts were seeded onto transfected HeLa cells, yielding a heterogeneous human-mouse cell population, followed by fusion of plasma membranes and subsequent immunofluorescent detection of protein-shuttling events. As evidenced by staining of DNA with DAPI, the

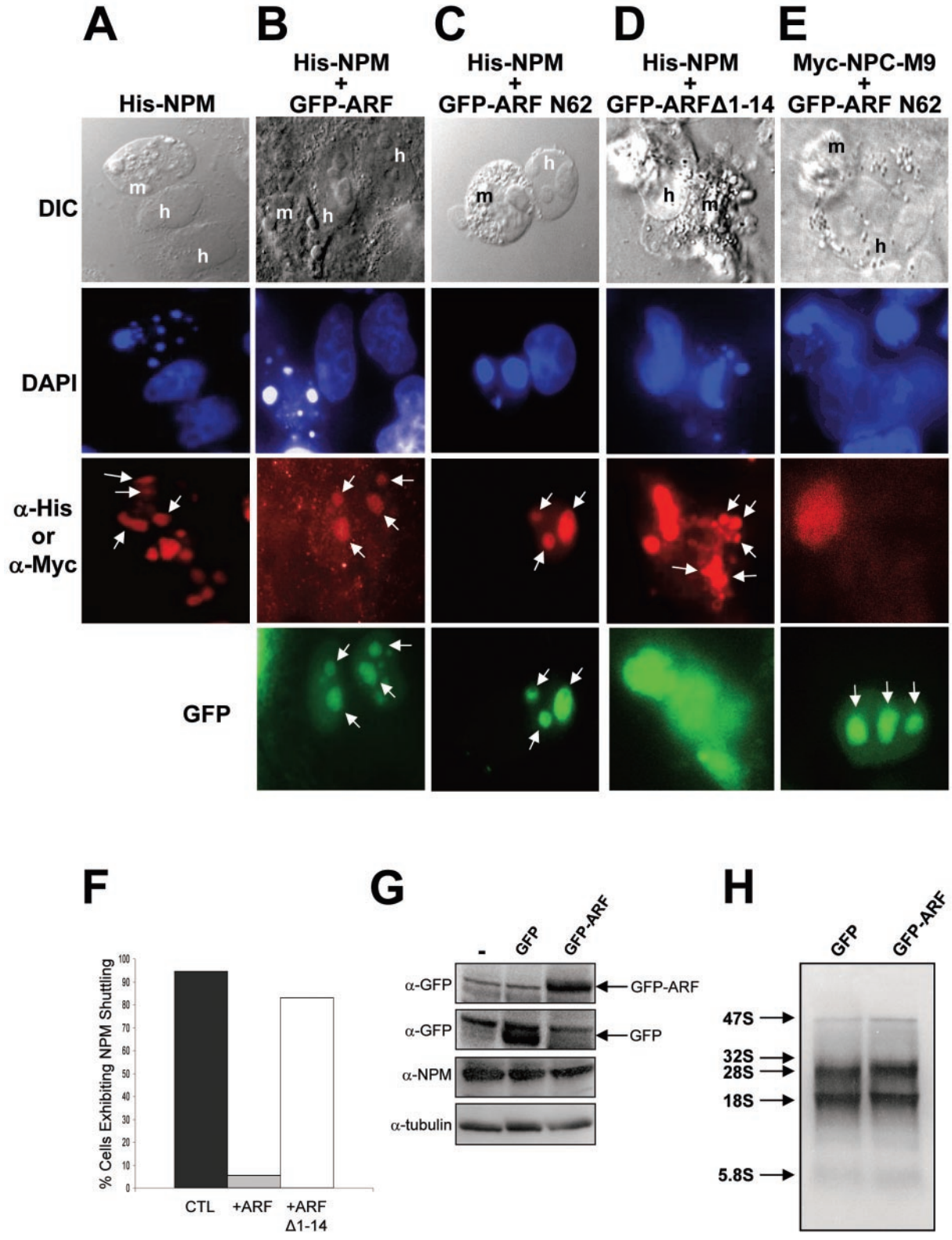

FIG. 6. ARF prevents the nucleocytoplasmic shuttling of NPM. NIH 3T3 cells (*ARF* null) were seeded onto HeLa cells that had been transfected with (A) His-NPM in combination with (B) GFP-ARF, (C) GFP-ARF N62, or (D) GFP ARF Δ1-14. (E) Myc-tagged NPC-M9 was transfected in combination with GFP-ARF N62 as a shuttling control. NIH 3T3 and HeLa cell cocultures were incubated with cycloheximide for 30 min prior to membrane fusion with polyethylene glycol. Fused cells (heterokaryons) were incubated in medium containing cycloheximide for an additional 4 h before fixation. Heterokaryon formation was verified under phase-contrast microscopy using a conventional differential interference contrast (DIC) filter, while His-NPM and ARF proteins were visualized with antibodies against His (red [A to D]) or Myc (red [E])

nuclei of NIH 3T3 and HeLa cells are easily distinguished by the greater heterochromatin focus content of mouse cells (speckled pattern) (Fig. 6, DAPI). In the absence of GFP-ARF, NPM readily migrated from human nucleoli to mouse nucleoli, as visualized in interspecies heterokaryons (Fig. 6A). However, in the presence of nucleolar GFP-ARF or GFP-ARF N62, NPM failed to shuttle and was restricted to human nucleoli within heterokaryons (Fig. 6B and C). Conversely, GFP-ARF Δ1-14, devoid of the NPM binding domain, was unable to restrict NPM shuttling between human and mouse nucleoli (Fig. 6D). As GFP-ARF did not hinder the nucleocytoplasmic trafficking of Myc-NPC-M9, an hnRNP protein that readily shuttles (Fig. 6E) (31), our combined body of data indicates that ARF specifically binds and sequesters NPM into nucleolar ARF complexes to completely impede NPM nucleocytoplasmic shuttling (Fig. 6F). Inhibition of NPM shuttling was not due to reduction of NPM protein, as GFP-ARF had no effect on NPM protein expression in HeLa cells (Fig. 6G). Additionally, GFP-ARF did not inhibit rRNA processing of 28S, 18S, and 5.8S subunits in HeLa cells (Fig. 6H), indicating that NPM's roles in rRNA processing and nuclear export are distinct and separate events.

NPM expression is required for cell cycle progression. Previous investigations into NPM function have correlated NPM mRNA and protein levels with the cell's growth state (12, 17), and our data further indicate that NPM may be essential for S phase entry. Based on our finding that NPM shuttling is required for cell cycle progression, we utilized RNA interference methodologies to more directly assess the contribution of NPM to cell proliferation. TKO MEFs transduced with retroviruses encoding NPM-targeting siRNA duplexes (pSUPER-NPM RNAi) displayed a significant knockdown in NPM protein expression by 72 h postinfection (Fig. 7A) with no observed increase in apoptosis (data not shown). In contrast, the levels of γ-tubulin (Fig. 7A), as well as other nucleolar proteins (data not shown), remained unchanged, demonstrating the specificity of our siRNA targeting sequence. Additionally, a scrambled siRNA did not reduce NPM levels (Fig. 7A), underscoring the fact that NPM protein expression is not influenced by introduction of nonspecific RNAi molecules. Importantly, pSUPER-NPM RNAi-infected TKO MEFs that displayed reduced NPM protein expression were significantly impaired in their ability to enter S phase compared to cells infected with control viruses that exhibited normal levels of NPM (Fig. 7B). Given that the reduction of NPM protein expression and the inhibition of NPM's nucleocytoplasmic shuttling each trigger growth arrest in MEFs, we conclude that NPM is an essential player in the process of cell cycle progression.

DISCUSSION

The nucleolus was originally described as the cell's command center for ribosomal biosynthesis and assembly, with a host of proteins being implicated in these processes. Nucleoli are not constrained by a membrane but rather actively recruit and retain proteins via arginine-lysine-rich nucleolar localization signal domains, such as those encoded within ARF, Mdm2, and NPM (22, 25, 43). In recent years, we have come to appreciate that proteins can actively shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is not merely a static site of ribosome biogenesis. While the nuclear and nucleolar functions of ARF have been heavily debated, several groups have shown that ARF can utilize nucleocytoplasmic transport to negate Mdm2-mediated degradation of p53 in the cytoplasm; specifically, ARF prevents the nuclear export of Mdm2 by actively sequestering it in the nucleolus (6, 10, 25, 26, 41, 43). It is reasonable to speculate that ARF, in its capacity as a tumor suppressor, may employ a similar tactic to regulate other growth-promoting proteins.

The key to ARF's proficiency in arresting cell proliferation, irrespective of p53 status, resides within its extreme amino terminus, namely, the first 14 residues of the ARF amino acid sequence. In search of additional interacting partners of this domain, we have verified the formation of both endogenous and exogenous ARF-NPM nucleolar complexes in primary mouse fibroblasts. Notably, ARF utilizes these exact residues to establish its high-affinity association with Mdm2, raising the possibility that ARF may affiliate with two distinct nucleolar complexes and/or represent a source of competition between Mdm2 and NPM. Upon its induction by hyperproliferative signals, ARF readily draws both Mdm2 and NPM into seemingly independent, distinct nucleolar complexes, evidenced by the absence of a ternary complex. However, overexpression of Mdm2 results in the release of NPM from ARF-containing protein complexes, suggesting that ARF's ability to dictate growth arrest in the presence or absence of p53 is largely determined by the stoichiometry of its binding partners. In light of this finding, it is appealing to think of Mdm2 as a target of ARF suppression, as well as a dampener of persistent ARF function. Specifically, Mdm2 may negatively regulate the p53 response through a dual mechanism: degradation of its direct transcriptional activator, p53 (38, 40), and inhibition of ARF function via preferential binding over NPM. We show here that Mdm2 can antagonize both the p53-dependent and -independent functions of ARF. By uncovering a pathway through which Mdm2 can promote cell growth independently of its classic upstream activator, p53, our study has exposed an unforeseen capacity of the Mdm2 oncoprotein.

and naturally emitting GFP spectra (green) using tetramethyl rhodamine isothiocyanate and FITC filters, respectively. The arrows indicate the staining pattern of nucleoli. The data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments with the aggregate NPM shuttling events of all experiments plotted (F). HeLa cells transfected with GFP or GFP-ARF were harvested and lysed 48 h after transfection. (G) Proteins were separated by SDS-PAGE and immunoblotted with antibodies recognizing γ-tubulin, NPM, and GFP. (H) For analysis of rRNA processing, transfected cells were labeled with [5,6-^{3}H]uridine for 45 min, followed by a 1-h chase. RNA was harvested and separated on agarose-formaldehyde gels, transferred to membranes, and visualized by autoradiography. The arrows indicate sizes of rRNA components.

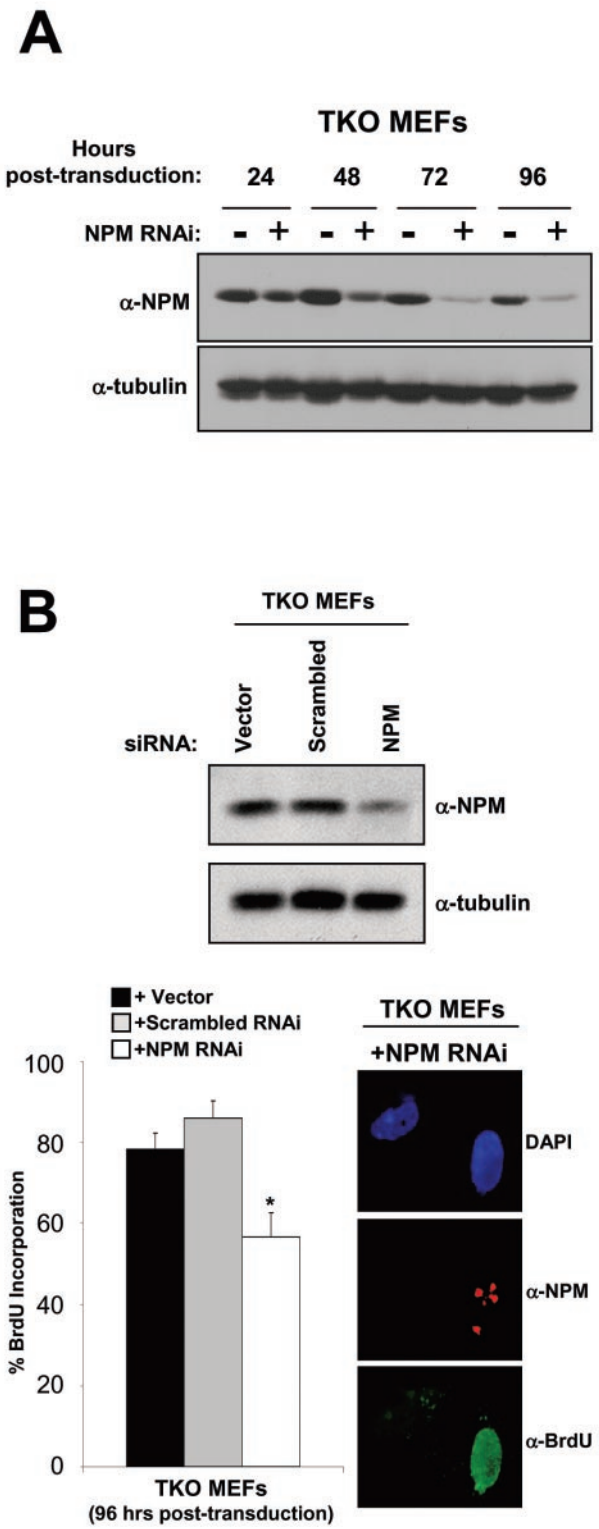

FIG. 7. Loss of NPM hinders cell cycle progression. (A) TKO MEFs infected with retroviruses encoding NPM-targeting siRNA duplexes (NPM RNAi) were harvested and lysed at 24-, 48-, 72-, and 96-h time points postinfection; as a control, uninfected cells were harvested and lysed in parallel. Whole-cell lysates were separated on denaturing polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM and γ-tubulin (loading control). (B) TKO MEFs transduced with either empty vector (dark bar), scrambled (shaded bar), or NPM (open bar) RNAi-encoding retroviruses were seeded onto 100-mm-diameter dishes and glass coverslips at 24 h postinfection, followed by addition of BrdU (10 μM) at 72 h postinfection (for coverslips only). At 96 h postinfection, the cells were either harvested for Western blot analysis (NPM and γ-tubulin) or fixed, and subjected to indirect immunofluorescent detection of NPM expression (red) and BrdU incorporation (green) using the antibodies described above. Nuclei were demarcated with DAPI. The error bars indicate standard deviations of 100 cells counted three times for each slide (*, $P > 0.005$).

While NPM is an abundant nucleolar phosphoprotein, data from our laboratory and others indicate that it is distributed throughout the cell in discrete pools, and factors such as NPM's state of posttranslational modification, bound protein partners, and subcellular localization may determine the composition and activity of any given NPM pool (5, 8, 22, 23, 30, 35, 47). As revealed by our data and the results of others, we hypothesize that ARF sequesters a specific pool of NPM in the nucleolus, preventing its transit and intended function(s) elsewhere in the cell. The intrinsic growth-promoting potential of the NPM pool immobilized within the ARF complex remains unclear, but recent studies support several possibilities. ARF transduction into MEFs lacking *ARF* and *p53* (*ARF*/*p53*-null) was shown to significantly impair the processing of rRNAs, and this effect was strictly dependent upon the highly conserved first 14 amino acids within ARF's extreme amino terminus (39). Importantly, transduction of p53 failed to reproduce this result (39), indicating that ARF's ability to down-regulate rRNA processing is distinct from ARF's established roles within the classical *ARF*/*p53*/*Mdm2* epistatic pathway. Additionally, two studies recently reported that NPM was a nucleolar ARF binding partner (4, 15). Both studies indirectly demonstrate that ARF can prevent proper rRNA processing, but how this might affect proper protein translation and how this event signals proliferative arrest remain unanswered. Nonetheless, these findings, in combination with our data, suggest that ARF's interaction with NPM may facilitate contact between ARF and the nucleolar ribosomal processing machinery, given that NPM appears to function as an integral component of ribosome maturation and export (30). We have further shown that NPM shuttling is required for cell proliferation, suggesting that ARF, via nucleolar sequestration of NPM, may not only target rRNA processing but might also prevent the nucleolar or nuclear export of processed rRNAs. This would be analogous to an earlier hypothesis in which protein targets of the ARF tumor suppressor may "ride the ribosome" out of the nucleolus and into the cytoplasm to execute their growth-promoting functions (37).

In an unrelated study, NPM was shown to be phosphorylated by cyclin E/cdk2 at the centrosome, resulting in the initiation of centrosome duplication (29). Hence, it is possible that nucleolar ARF, in response to hyperproliferative signals, binds and immobilizes the NPM pool that is designated for transit to the centrosome, consistent with our findings. However, a more recent report failed to detect NPM in isolated centrosomes (1), raising the possibility that other functional targets of NPM reside within or are transported to the cytoplasm, one of which might be the maturing ribosome itself. The models described above warrant further investigation, but we cannot overlook

the possibility that NPM may, in addition to its ascribed roles in ribosome biogenesis and centrosome duplication, transmit additional growth-promoting commands and that Mdm2-mediated release of NPM from ARF complexes potentiates these functions. Nonetheless, ARF, in its capacity as a tumor suppressor, employs redundant mechanisms of protein binding and topological sequestration to inhibit both p53-dependent and -independent targets, thereby achieving control over cell proliferation.

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Nucleophosmin is essential for ribosomal protein L5 nuclear export

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ABSTRACT

Nucleophosmin (NPM/B23) is a key regulator of three important cellular processes: centrosome duplication, genomic integrity and ribosome biogenesis. Although the mechanisms behind these NPM functions are largely unknown, it is evident that loss of *NPM* results in severe dysregulation of each process, underscoring the importance of NPM in maintaining cell well-being. We show that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino-terminus to actively shuttle between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically isolated NPM-bound proteins from HeLa cell lysates. Consistent with a proposed role in ribosome biogenesis, we isolated two ribosomal proteins, rpL22 and rpL5, that aid in the assembly of the mature 60S ribosomal subunit. Direct interaction of NPM with rpL5 proteins allowed for the co-localization of NPM with maturing nuclear 60S ribosomal subunits as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of *NPM* completely blocked the nuclear export of rpL5, suggesting that NPM provides a unique chaperoning activity towards rpL5 as it is exported to the cytosol.

INTRODUCTION

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site for active transcription of ribosomal RNAs (rRNA) and ribosome assembly (7). Various nucleolar proteins, RNAs, and other factors have been suggested to be involved in this complex process of ribosome production and maturation (17). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 40). While the nucleolar proteome contains many proteins and ribonucleoproteins proposed to be involved in ribosome biogenesis, a remarkable number of proteins identified (>100) have no known function. The difficulty in assessing nucleolar protein function stems from early assumptions that all nucleolar proteins must be involved, in some way, with static ribosome biogenesis by virtue of their unique subcellular localization. However, a more contemporary view of the nucleolus as a dynamic nuclear organelle capable of regulating numerous cellular processes has led to a re-evaluation of nucleolar protein function(s) (25).

Nucleophosmin (NPM/B23) is an abundant phosphoprotein localized in the granular regions of the nucleolus (42). NPM was found to be highly expressed in proliferating cells (8, 12), and has been associated with a variety of cellular phenomena, including ribosomal biogenesis, protein chaperoning and centrosome duplication (12, 22, 31, 32). Structurally, NPM can exist in both a monomeric and multimeric state, although NPM multimers seem to dominate in the nucleolus and may be crucial for the assembly of maturing ribosomes (29, 30, 49). More importantly, NPM, along with other nucleolar proteins, has been suggested to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may contribute to some of its essential

functions (5). Indeed, NPM exit from the nucleolus/nucleus is an essential event in S phase progression; inhibition of this trafficking by the nucleolar tumor suppressor ARF results in cell cycle arrest (6). Additionally, NPM is an essential nucleolar protein with loss of its expression resulting in severe attenuation of cellular proliferation and increased apoptosis (4, 6, 15, 18), underscoring NPM's importance to the cell.

If nuclear exit of NPM plays a positive role in promoting cell growth and proliferation, what necessary function is it performing? While numerous proteins, such as Mdm2, cdc14p and TERT, are topologically restrained in the nucleolus following defined cellular cues, synthesis and export of newly synthesized ribosomal subunits from the nucleolus remains the only known nucleolar-specific event conserved throughout evolution (41). Recent work from *Xenopus laevis* and *Saccharomyces cerevisiae* has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (19) as well as a nuclear adaptor protein NMD3 that is conserved from yeast to man (47). In an effort to uncover the biological significance of NPM protein trafficking, we first discovered that NPM's exit from the nucleus also involved the classical CRM1-dependent nuclear export pathway. In search of targets for NPM export regulation, we discovered the ribosomal protein rpL5, a 60S subunit protein that chaperones the 5S ribosomal RNA into the nucleolus and out into the cytosol (28), was a direct binding partner for cytosolic and nuclear NPM proteins. We propose that NPM helps direct rpL5 nuclear export through a CRM1-dependent mechanism, providing NPM with direct access to the maturing ribosome and potential regulation of the translational machinery.

MATERIALS AND METHODS

Cell Culture.

HeLa and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids, and 100 U penicillin and streptomycin.

Plasmid Constructs.

Vectors encoding full-length His-tagged murine NPM are described elsewhere (6). His-epitope tagged NPM was subcloned into pcDNA3.1 (Invitrogen) and pEGFP (Clontech) vectors. His-NPM$_{\Delta 42\text{-}61}$, His-NPM$_{\Delta 62\text{-}83}$ or His-NPMdL mutants were generated using the following primers: 5'-GAAAATGAGCACCAGGCAGAAGC AATGAAC-3' (sense) and 5'-GTTCATTGCTTCTGCCTGGTGCTCATTTTC-3' (antisense), 5'-GTTAC ACATCGTAGAGCAACCAACAGTTTCC-3' (sense) and 5'-GGAAACTGTTGGT TGCTCTACGATGTGTAAC-3' (antisense), or 5'-GAAAATGAGCACCAGGCGTC AGCAAGAACGGTC-3' (sense) and 5'-CTAAACTGACCGTTCTTGCTGAC GCCTGGTGCTCATTTTC-3' (antisense), respectively using QuickChange Mutagenesis (Stratagene). A myc-tagged NPC-M9 (36) in pcDNA3 and a GFP-tagged rpL5 plasmid (37) were generous gifts from Alan Diehl (University of Pennsylvania), and Joachim Hauber (Universitat Erlangen-Nurnberg).

Heterokaryon Assay.

HeLa cells ($2x10^5$) were seeded onto glass cover slips and transfected with plasmids as indicated. NIH3T3 cells ($6x10^5$) were seeded onto the HeLa cells 24 h post-transfection.

Co-cultures were then incubated for 30 min with cyclohexamide (100 μg/ml) followed by incubation with 50% polyethylene glycol (PEG) in PBS for 105 sec. Co-cultures were incubated with DMEM containing cyclohexamide (100 μg/ml) for an additional 4 h. Heterokaryons were fixed and stained with a rabbit anti-His antibody (Santa Cruz), or mouse anti-myc antibody (Zymed), followed by either fluorescein isothiocyanate (FITC)-conjugated or Rhodamine X-conjugated anti-rabbit or anti-mouse immunoglobulin (Pierce) as described (6). Nuclei were stained with Hoechst (Sigma). Fluorescent signals were detected using a Nikon epifluorescent compound microscope (100X) fitted with a Nikon FDX-35 camera.

Immunoprecipitation and Western Blot Analysis.

Cells were transduced with vectors encoding His-NPM, His-NPMdL, and GFP-rpL5 and lysed in binding buffer (25 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) 48 h after nucleofection as recommended by the manufacturer (Amaxa). Primary antibody to the NPM N-terminus (rabbit, Sigma Genosys), GFP (Santa Cruz), His (Santa Cruz), rpL5 (11) or non-immune rabbit serum (NRS) was added to the binding reactions. Immune complexes were precipitated with protein A-Sepharose (Amersham). The precipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes. NPM, His-tagged proteins and GFP-tagged proteins were visualized by direct immunoblotting with NPM (Zymed), His (Santa Cruz), rpL5 and GFP (Santa Cruz) antibodies, respectively.

Fluid Phase Liquid Chromatography.

HeLa cells were lysed in Tween-20 lysis buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20; 1 μM $NaVO_4$; 10 μM NaF; 1 mM PMSF; 1 μg/ml Aprotinin) by sonication. Lysates (600 μg) were injected on to a HiPrep 16/60 Sephacryl S-300 Column (Amersham). Proteins were eluted with 150 mM NaCl, 50 mM NaH_2PO_4 pH 7.2 using BioLogic fluid phase liquid affinity chromatography (FPLC) and HR software (Bio-Rad). Fractions were precipitated with trichloroacetic acid (TCA), re-suspended in 1 M Tris-HCl (pH 7.4), separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies recognizing NPM (Zymed). For affinity chromatography, a rabbit polyclonal antibody recognizing the N-terminus of NPM (Sigma) was bound to NHS-activated Sepharose (Amersham). HeLa cells were lysed in 20 mM Tris pH 7.4, 0.1% Tween-20 and sonicated. Lysates (600 μg) were injected onto the NPM affinity column, washed with 20 mM Tris and eluted with an increasing NaCl gradient (0.1 to 1M). Fractions were collected and analyzed as above.

Proteomic Analysis.

Proteins from FPLC fractions were precipitated with TCA and resuspended in Laemmli buffer. SDS-PAGE separated proteins were stained with SYPRO-Ruby (BioRad). Bands of interest were excised and processed for trypsin digestion. Tryptic peptides were calibrated with Sequazyme peptide mass standards kit (PE Biosystem) and analyzed by MALDI-TOF mass spectrometry (Voyager DE Pro, Applied Biosystems). Identification of proteins was performed using MS-Fit software (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). MALDI-TOF spectra and sequences

were verified using a 4700 Proteomics MS/MS system (Applied Biosystems). Identified proteins were additionally verified by direct western blot analysis.

Subcellular fractionation.

HeLa cells were transfected with scrambled or siNPM RNAs and harvested. Pellets containing equal cell numbers were resuspended in HEPES buffer (10 mM HEPES, pH 7.4 with 4mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1μg/ml pepstatin) and lysed with a syringe. Lysates were pelleted and the supernatant was saved as the cytoplasmic fraction. The pellet was resuspended in fractionation buffer (10 mM Tris pH7.5, 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 4mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1μg/ml pepstatin), dounced, layered over a cushion of sucrose (45% w/v in fractionation buffer), and centrifuged. The pellet was washed and resuspended in EBC buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1μg/ml pepstatin, 1 mM NaF, 10 mM $NaVO_4$, β-glycerophosphate). Nuclear or cytoplasmic protein was subjected to SDS-PAGE. SOD (Cu/Zn specific form), lamin A/C, and rpL5 proteins were visualized by direct immunoblotting with anti-SOD (Calbiochem); anti-lamin A/C (Santa Cruz), and anti-rpL5 antibodies (11), respectively.

Ribosome Fractionation

Cells were subjected to cytosolic and nuclear ribosome fractionation and lysates were separated on sucrose gradients as previously described (44). RNA was continuously monitored over the gradient by ultraviolet absorbance at 254 nm. Fractions were

collected and proteins were precipitated with TCA. Proteins were separated by SDS-PAGE immunoblotted with antibodies recognizing NPM (Zymed) and rpL5.

RESULTS

NPM Nuclear Export Requires a CRM1-Dependent Nuclear Export Signal Involving Leucines 42 and 44.

NPM is a ubiquitously expressed nucleolar phosphoprotein capable of regulated nuclear import (5). When NPM is transiently expressed in mammalian cells, it localizes predominantly to the nucleolus. Moreover, using *in vivo* heterokaryon shuttling assays (46), we have previously shown that NPM readily shuttles between the nucleolus/nucleus and cytoplasm (6). NPC-M9, a nuclear hnRNP protein that readily mobilizes to the cytoplasm, serves as a shuttling control (36). To distinguish between human donor and murine acceptor nuclei, chromosomal DNA was stained with Hoechst, clearly demarcating greater heterochromatin foci of NIH 3T3 mouse cells (speckled pattern, Fig. 1, Hoechst). As shown in Fig. 1A, NPM readily shuttles out of the human nucleolus, into the fused cytoplasm and back into the mouse acceptor nucleus/nucleolus.

Given that a wide range of shuttling proteins utilize the CRM1 transport protein for their nuclear export, we further investigated the underlying export mechanism of NPM both in the presence and absence of leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export (23). In the absence of LMB, NPM readily migrated from human nucleoli to mouse nucleoli (Fig. 1A). However, in the presence of LMB, NPM failed to shuttle, and was restricted to human nucleoli within heterokaryons (92% inhibition, Fig. 1B). The addition of LMB did not hinder the nucleocytoplasmic

trafficking of Myc-NPC-M9, an hnRNP that readily shuttles in a CRM1-independent nuclear export pathway (34).

A sequence alignment of NPM residues with known CRM1-dependent shuttling proteins revealed two motifs containing short leucine rich hydrophobic stretches of amino acids characteristic of CRM1-dependent nuclear export sequences (NESs) (Fig. 1C) (13, 14). In order to identify which regions of NPM contain its NES, we generated deletion mutants of NPM lacking either of the two potential NESs, NPM Δ42-61 or Δ62-83. Using these NPM constructs, we again conducted interspecies heterokaryon assays. As shown in Fig. 2A, deletion of amino acids 42-61 of NPM (His-NPM$_{\Delta 42\text{-}61}$) prevented its shuttling (100% inhibition) to mouse nucleoli. Importantly, a myc tagged-NPC-M9 shuttling control readily shuttled in the same human-mouse heterokaryon, indicating that these heterokaryons were not impaired for nucleocytoplasmic shuttling in general. In contrast, deletion of amino acids 62-83 of NPM (His-NPM$_{\Delta 62\text{-}83}$) did not prevent NPM's ability to shuttle between human and mouse nucleoli (6% inhibition, Fig. 2B), revealing that the putative NES resides within amino acids 42-61 of the NPM protein.

Since the type of NES recognized and bound by the CRM1 export receptor is dependent on closely spaced hydrophobic amino acids (particularly leucines) (13, 14), we introduced point mutations into the corresponding leucine residues within the NES of NPM (Leu-42 and Leu-44 to Ala-42 and Ala-44). First, we tested this NPM mutant (designated NPMdL for double Leucine mutant) with Myc-NPC-M9 as a shuttling control. As expected, NPMdL was unable to transit from a human nucleus to the cytoplasm and into a murine nucleus (100% inhibition), indicating that these two leucines are critical for nuclear export of the NPM protein (Fig. 2C). Sequence alignment of

numerous nucleophosmin homologues underscores the evolutionary importance of this amino-terminal export motif as it is nearly identical from zebrafish to man (Fig 2D).

Heterogeneous NPM NES Mutants and Wild-Type NPM Complexes Fail to Shuttle.

Because NPM readily self-oligomerizes when active in the nucleolus (49), we examined whether the NPM shuttling mutant, NPMdL, could also block wild-type NPM from shuttling. In the absence of the shuttling mutant, green fluorescent protein (GFP)-tagged NPM readily shuttled from human to mouse nucleoli (Fig. 3A). However, in the presence of His-tagged NPMdL, GFP-NPM was retained in human nuclei (Fig. 3B, 96% inhibition). To establish the mechanism behind this finding, we performed co-immunoprecipitation experiments to determine whether mutant NPM molecules could oligomerize with wild-type NPM proteins *in vivo*. As seen by western blot analysis, His-NPMdL complexes contained significant quantities of endogenous wild-type NPM proteins, indicating that *in vivo*, mutant NPM readily oligomerizes with wild-type NPM to prevent its shuttling (Fig. 3C).

NPM Associates with Cytoplasmic and Nuclear rpL5 Ribosome Complexes.

Previous studies have indicated that NPM might function as an integral component of ribosome maturation through its RNA binding activities (32). However, most hypotheses in this regard are largely based on the fact that NPM is nucleolar, and thus, most likely to be involved in the major process in the nucleolus: ribosome biogenesis. To formally test the nucleolar function of NPM, we examined the composition of *in vivo* NPM protein complexes in HeLa cell lysates. We generated a

custom NPM polyclonal antibody affinity column and used a control non-immune immunoglobulin column to pre-clear protein lysates. NPM complexes were eluted with increasing salt concentrations and visualized following SDS-PAGE and SYPRO-Ruby staining (Fig. 4A). Protein bands were excised and identified using MALDI-TOF and MS/MS analyses. Among those proteins bound to NPM, a cluster of proteins associated with ribosome biogenesis including rpL5, rpL22 and nucleolin, as well as the nuclear pore complex proteins, Nup50 and Nup62, were identified (Fig. 4A and B). Western blot analysis of NPM protein complexes verified the presence of NPM and rpL5 in salt-eluted fractions (Fig. 4C).

Having identified two members of the 60S ribosomal subunit, namely rpL22 and rpL5 (Fig. 4B), in NPM complexes, we wanted to evaluate the localization of NPM with ribosomes *in vivo*. Co-immunoprecipitation of endogenous NPM and rpL5 from HeLa cells again verified the formation of NPM-rpL5 complexes *in vivo* (Fig 5A, left panel). Moreover, NPM and rpL5 formed direct complexes *in vitro* using purified NPM and rpL5 proteins, and importantly, this binding occurred in the presence of wild-type and NES mutant NPM proteins (Fig. 5A). In order to follow the spatial control of NPM-rpL5 complexes *in vivo*, we utilized the UV absorbance of the ribosome. RpL5 is known to provide the maturing 60S ribosomal subunit with 5S ribosomal RNA prior to nucleolar/nuclear export of the 60S subunit (43), providing NPM an ideal time to form nucleolar complexes with rpL5. Cytoplasmic and nuclear extracts of HeLa cells were subjected to sucrose gradient centrifugation, and the gradients fractionated with continuous UV monitoring. As shown in Fig. 5B, NPM associates with the 40S, 60S, 80S, and polysome fractions in the cytoplasm while nuclear pools of NPM associate with

the 40S/Pre-60S and 60S fractions in the nucleus. Consistent with previous reports (27), we found rpL5 associated with the 60S, 80S, and polysome fractions in the cytoplasm, and the 40S/Pre-60S and 60S fractions in the nucleus (Fig. 5B). These data demonstrate that NPM and rpL5 form complexes with the maturing 60S ribosomal subunits in the nucleus and are maintained in the mature ribosome once it reaches the cytosol and also indicate that NPM might also associate with the 40S subunit which is devoid of rpL5 (Fig. 5B) (27).

NPM Is Required for rpL5 Nuclear Export.

Having demonstrated earlier the intracellular mobility of NPM and that NPM-rpL5 complexes are present in both the nucleus and cytosol, we next examined the influence of the NPM shuttling mutants on rpL5 nuclear export using a previously characterized GFP-tagged rpL5 protein (37). To confirm that GFP-rpL5 retained the NPM-binding properties of the endogenous rpL5 protein, we transiently overexpressed GFP-rpL5 in HeLa cells and performed western blot analysis of GFP-immunoprecipitated complexes. As shown in Fig. 6A, precipitated GFP-rpL5 complexes contained endogenous NPM, confirming that the GFP moiety does not adversely affect the formation of NPM-rpL5 complexes *in vivo*. GFP-rpL5 and His-NPM readily migrated from human nucleoli to mouse nucleoli, as visualized in interspecies heterokaryons (Fig. 6B). However, in the presence of LMB, both GFP-rpL5 and His-NPM failed to shuttle (95% inhibition, Fig. 6C). Introduction of two NPM shuttling mutants, $NPM_{\Delta 42\text{-}61}$, or NPMdL, inhibited GFP-rpL5 shuttling into mouse nucleoli, restricting its expression to human nuclei (Fig. 6D and 6E, 96% and 100% inhibition, respectively), establishing that

NPM nuclear export is required for the export of rpL5. To more definitively show that NPM is required for rpL5 nuclear export, we completely knocked-down NPM expression in HeLa cells (Fig. 7A). Cells lacking NPM protein expression failed to accumulate rpL5 in the cytoplasm while cells transduced with scrambled siRNA as a control exhibited an equal distribution of rpL5 between the nucleus and cytoplasm (Fig. 7B). These data underscore the necessity of NPM proteins for the efficient transport of rpL5 out of the nucleus and into the cytoplasm.

DISCUSSION

The nucleolus, a highly specialized and structured organelle, has been described as the cell's control center for ribosomal synthesis, maturation, and assembly, with a host of proteins, RNAs, and other elements being implicated in these processes (7). Numerous proteins (cdc14, NPM, cyclin E, Mybbp1a, TERT, etc.) have been recently shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of numerous cellular events (3, 6, 20, 21, 48).

One of these nucleolar proteins, NPM/B23, has been suggested to be involved in a variety of important cellular processes in and out of the nucleolus, including ribosome processing, molecular chaperoning, genomic integrity, centrosome duplication, and transcriptional regulation (8, 9, 12, 15, 22, 31). Notably, NPM imported into the nucleolus from the cytoplasm was initially presumed to move about within various compartments of the nucleus (5), a feature shared by many critical cell cycle regulators.

It has become well-established that shuttling between the nucleus and cytoplasm provides a critical layer of control over cell cycle progression (35, 39). In previous reports, we and others identified NPM as a novel p53-independent target of the ARF tumor suppressor protein (4, 6, 18). We have since shown that interactions between ARF and NPM, in response to hyperproliferative signals, result in the inhibition of NPM's nucleocytoplasmic shuttling capabilities. Here, we have further explored the mechanism and significance of NPM intracellular trafficking. First, we have described the CRM1-dependent nuclear export of NPM, identifying the two critical leucine residues (42 and 44) involved in this process. In addition, alterations within the NPM NES resulted in the failure of wild-type NPM to export out of the nucleolus providing evidence that such mutations act in a dominant fashion presumably through the formation of NPM-NPMdL hetero-multimers. Thus, NPMdL mimics the effects of ARF induction by impeding the nucleocytoplasmic shuttling of NPM through direct interaction, underscoring the overall importance of NPM exit from the nucleolus/nucleus in maintaining cell growth.

We had previously suggested that targets of nucleolar sequestration might in fact "ride the ribosome" from the nucleolus to the cytoplasm to perform growth promoting functions (41). In agreement with this hypothesis, our findings reveal a direct interaction between NPM and rpL5, providing the first evidence to physically link NPM with ribosomal subunits. Although much of the focus in the field has been on the potential function of rpL5 to deliver 5S rRNA to the nucleolus after initial transcription of 5S rRNA by RNA polymerase III in the nucleoplasm (28, 33, 43), it is also possible that rpL5 plays a critical role in the export of large ribosomal subunits (60S) containing 5S rRNA from the nucleolus/nucleus to the cytoplasm after its assembly. These latter events

clearly would render themselves sensitive to regulation by NPM which provides the necessary export signals and chaperoning capabilities (through its interaction with rpL5) required to transport components of the ribosome to the cytosol. Inhibition of NPM nuclear export via deletion or mutation of its NES prevented the trafficking of rpL5, an integral component of the 60S ribosomal subunit. Moreover, ablation of NPM expression through RNA interference resulted in a complete lack of rpL5 in the cytoplasm, underscoring the absolute requirement of NPM for rpL5 nuclear export. While many components of the ribosome, including rpL5, contain their own NES, it is clear that a single NES bound to CRM1 is quite weak (24), suggesting that additional NESs are required for efficient complex export and that proteins like NPM and NMD3 may have evolved to serve this purpose. Considering that a major function of rpL5 is the binding and nucleolar/nuclear transport of 5S rRNA molecules, it remains to be seen what effect, if any, NPM shuttling has on 5S rRNA nuclear export. However, we did find NPM in ribosome complexes both in the nucleus and cytoplasm, implying that NPM, bound to rpL5, remains associated with the mature ribosome as it assembles in the cytosol and forms actively translating polysomes. This opens up the possibility that NPM might provide additional functions (outside of nuclear export) to cytosolic ribosomes during translation which would be consistent with proposed roles of the nucleolus in setting translation rates (25).

While it has been appreciated for several decades that changes in nucleolar structure are reliable markers of cellular transformation, little has been done to investigate a direct link between nucleolar dysfunction and tumorigenesis. In fact, the nucleolus has always been dismissed as a static organelle with no impact on overall cell

well-being. However, this “nucleolar stigma” has recently been challenged with the discovery that tumor suppressors, such as p53 and ARF, play a direct role in regulating nucleolar processes (4, 6, 38, 45). Interestingly rpL5 is also a binding partner of Mdm2 and p53 (11, 16, 26), implying that rpL5 may provide an intriguing mechanistic link between ARF and its binding partners. Indeed, NPM itself is a unique player in both the p53 and ARF responses (9, 10). Our findings demonstrate that NPM plays a direct role in regulating rpL5 nuclear export, a process that, given NPM’s interactions with ARF and p53, may lend itself sensitive to oncogenic and tumor suppressive signals.

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FIGURE LEGENDS

FIG. 1. Nuclear export of NPM is CRM1-dependent. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with His-NPM in combination with Myc-NPC-M9 (shuttling control) in the (A) absence or (B) presence of leptomycin B (LMB). Heterokaryons were incubated in media containing cyclohexamide for an additional 4 h before fixation. Heterokaryon formation was verified under phase contrast microscopy, while His-NPM and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dashed circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentage of His-NPM shuttling in heterokaryons is given. (C) Sequence alignment of putative NPM nuclear export sequences (NESs) with known NESs of CRM1-dependent nuclear export proteins (p53, PKI, rev, and Mdm2). Critical hydrophobic residues are indicated in yellow.

FIG. 2. Leucine-42 and leucine-44 are identified as critical nuclear export residues. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with (A) His-NPM$_{\Delta 42\text{-}61}$, (B) His-NPM$_{\Delta 62\text{-}83}$, or (C) NPMdL in combination with Myc-NPC-M9. Ectopic NPM proteins and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dashed circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each

transfection condition in three independent experiments. The percentage of His-NPM shuttling in heterokaryons is given. (D) Sequence alignment of NPM homologues throughout evolution. Identical residues in all species are marked yellow, identical residues in at least seven species are highlighted blue and conserved residues are marked green. The consensus NPM sequence for all eleven identified homologues is given with conserved nuclear export leucines 42 and 44 marked with arrows.

FIG 3. NPM shuttling mutants act as dominant negative inhibitors of NPM nuclear export. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with (A) GFP-NPM alone or (B) in combination with His-NPMdL**.** Heterokaryon assays were performed and His-NPMdL and GFP-NPM proteins were visualized with antibodies against His (red) or naturally-emitting GFP spectra (green). DNA was stained with Hoechst. Mouse nuclei are demarcated with dashed circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentage of GFP-NPM shuttling in heterokaryons is given. (C) HeLa cells transfected with His-NPMdL were lysed and the whole cell lysate subjected to immunoprecipitation with non-immune rabbit serum (NRS) or antibodies recognizing His epitopes. Precipitated protein complexes were separated by SDS-PAGE and ectopic mutant and endogenous wild-type NPM proteins were visualized with antibodies against NPM. Untransfected HeLa whole cell lysate was loaded as a marker for endogenous NPM expression (lane 1).

FIG 4. Isolation of endogenous NPM protein complexes. (A) HeLa cell lysates (600 μg) were injected onto a custom NPM polyclonal antibody affinity column and eluted with an increasing NaCl gradient (0.1-1.0M). Eluted proteins were separated by SDS-PAGE and visualized with SYPRO-Ruby dye. Identified bands are labeled. (B) Representative MALDI-TOF spectra of labeled protein bands from above are shown with labeled matching peptide masses. (C) NPM complexes from HeLa lysates eluted from the affinity column were separated by SDS-PAGE and immunoblotted with antibodies recognizing NPM and rpL5 proteins.

FIG 5. NPM interacts directly with rpL5 in nuclear and cytosolic ribosome complexes. (A, left panel) Proteins from HeLa cell lysates were immunoprecipitated (IP) with non-immune rabbit serum (NRS), rabbit rpL5 antibody (L5) or a rabbit NPM antibody. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with NPM and rpL5 antibodies. (A, center and right panels) His-NPM, His-NPMdL and His-rpL5 proteins were expressed and purified from bacteria using nickel affinity chromatography. Pure NPM or NPMdL proteins were incubated overnight with rpL5 and immunoprecipitated with NRS or antibodies recognizing NPM or rpL5. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with NPM and rpL5 antibodies. (B) HeLa cells were divided into cytoplasmic and nuclear fractions and subjected to sucrose gradient centrifugation. Absorbance was monitored at 254 nm and fractions containing 40S, 60S, 80S and polysome units were collected. Proteins from each fraction were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies recognizing

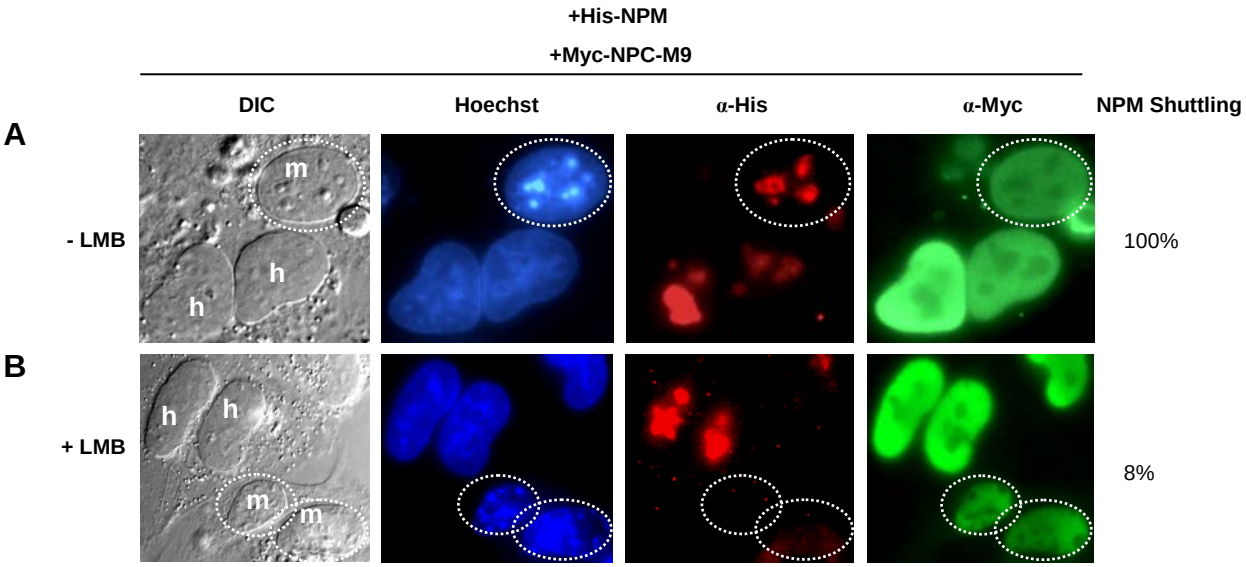
NPM and rpL5. Western blot lanes are placed above corresponding ribosome absorbance measurements.

FIG 6. NPM nuclear export signals are required for the efficient export of GFP-rpL5. (A) HeLa cells either untransfected or transfected with GFP-tagged L5 for 48 hours were harvested and lysed. Proteins were immunoprecipitated (IP) with non-immune rabbit serum (NRS), or a rabbit GFP antibody. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with GFP and NPM antibodies. Loading inputs are indicated. (B-E) NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-rpL5 in combination with (B and C) His-NPM, (D) His-NPM$_{\Delta 42\text{-}61,}$ and (E) His-NPMdL$_{.}$ Additionally, HeLa cells in (C) were treated with LMB for 18 h prior to fusion. Heterokaryon assays were performed with NPM and GFP-rpL5 proteins being visualized with antibodies against His (red) and naturally-emitting GFP spectra (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dashed circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed in three independent experiments. The percentage of heterokaryons exhibiting GFP-rpL5 shuttling is given.

FIG 7. NPM is essential for rpL5 nuclear export. (A) HeLa cells (-) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 hours post-transduction for western blot analysis. Proteins separated by SDS-PAGE were transferred to PVDF membranes and immunoblotted with antibodies

recognizing NPM and γ-tubulin. (B) HeLa cells (-) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 hours post-transduction for cellular fractionation. Proteins from nuclear (N) and cytosolic (C) fractions were analyzed by SDS-PAGE and immunoblotted with antibodies recognizing rpL5, SOD (cytoplasm control), and lamin A/C (nuclear control).

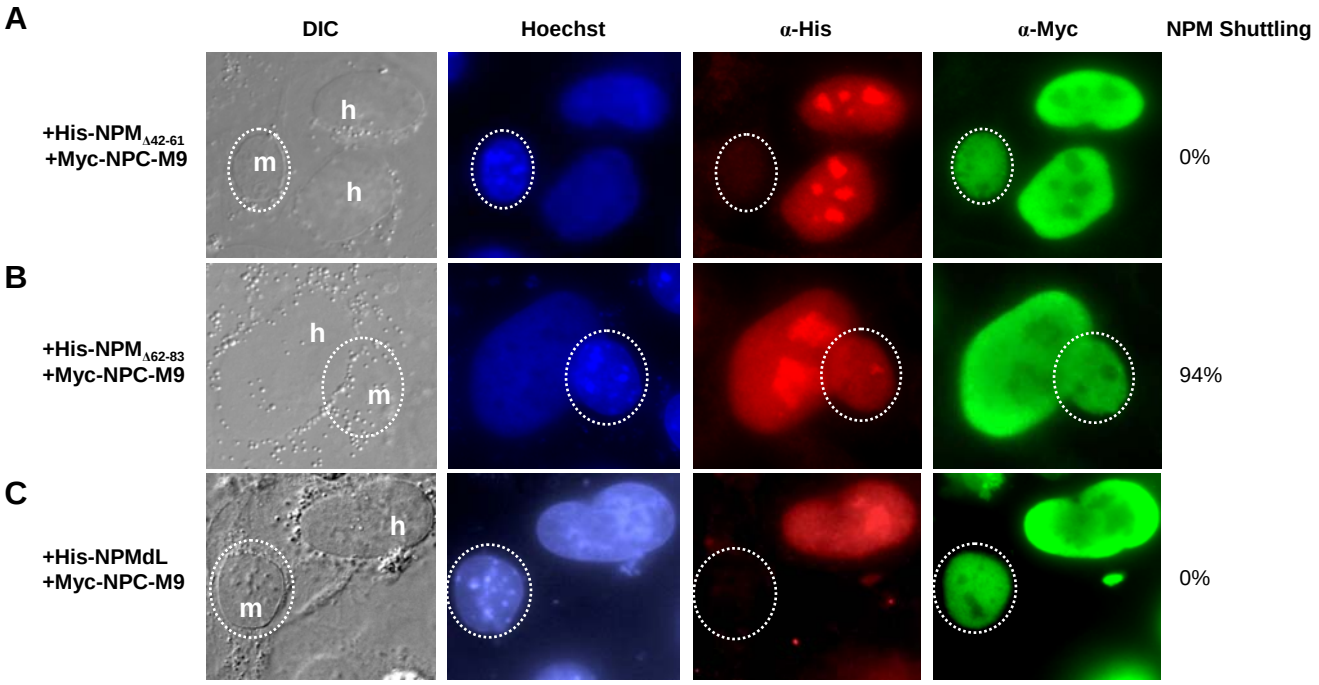
Figure 1



C

p53:	337	**M**	F	R	E	**L**	N	E	A	**L**	E	**L**	347
PKI:	37	**L**	A	**L**	K	**L**	-	A	G	**L**	D	**I**	46
HIV rev:	75	**L**	P	-	P	**L**	D	E	R	**L**	T	**L**	84
Mdm2:			184	**L**	S	**F**	-	E	S	**L**	A	**L**	191
NPM:			42	**L**	S	**L**	-	R	T	**V**	S	**L**	49
NPM:	72	**I**	K	**V**	T	**L**	-	A	T	**L**	K	**M**	81

Figure 2


A
DIC
Hoechst
α-His
α-Myc
NPM Shuttling
+His-NPMΔ42-61
+Myc-NPC-M9
h
m
h
0%
B
+His-NPMΔ62-83
+Myc-NPC-M9
h
m
94%
C
+His-NPMdL
+Myc-NPC-M9
h
m
0%

D

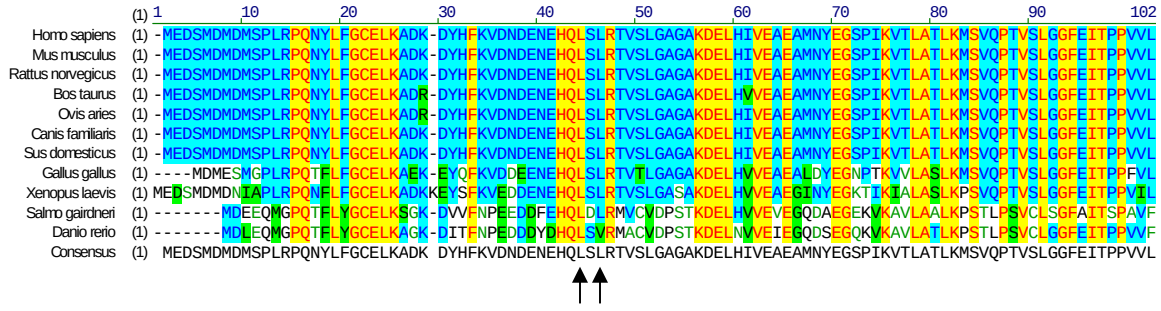

(1) 1 10 20 30 40 50 60 70 80 90 102
Homo sapiens (1) -MEDSMDMDMSPLRPQNYLFGCELKADK-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Mus musculus (1) -MEDSMDMDMSPLRPQNYLFGCELKADK-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Rattus norvegicus (1) -MEDSMDMDMSPLRPQNYLFGCELKADK-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Bos taurus (1) -MEDSMDMDMSPLRPQNYLFGCELKADR-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHVVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Ovis aries (1) -MEDSMDMDMSPLRPQNYLFGCELKADR-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Canis familiaris (1) -MEDSMDMDMSPLRPQNYLFGCELKADK-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Sus domesticus (1) -MEDSMDMDMSPLRPQNYLFGCELKADK-DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL
Gallus gallus (1) ----MDMESMGPLRPQTFLFGCELKAEK-EYQFKVDDEENEHQLSLRTVTLGAGAKDELHVVEAEALDYEGNPTKVVLASLKMSVQPTVSLGGFEITPPFVL
Xenopus laevis (1) MEDSMDMDNIAPLRPQNFLFGCELKADKKEYSFKVEDDENEHQLSLRTVSLGASAKDELHVVEAEGINYEGKTIKIALASLKPSVQPTVSLGGFEITPPVIL
Salmo gairdneri (1) -------MDEEQMGPQTFLYGCELKSGK-DVVFNPEEDDFEHQLDLRMVCVDPSTKDELHVVEVEGQDAEGEKVKAVLAALKPSTLPSVCLSGFAITSPAVF
Danio rerio (1) -------MDLEQMGPQTFLYGCELKAGK-DITFNPEDDDYDHQLSVRMACVDPSTKDELNVVEIEGQDSEGQKVKAVLATLKPSTLPSVCLGGFEITPPVVF
Consensus (1) MEDSMDMDMSPLRPQNYLFGCELKADK DYHFKVDNDENEHQLSLRTVSLGAGAKDELHIVEAEAMNYEGSPIKVTLATLKMSVQPTVSLGGFEITPPVVL

Figure 3

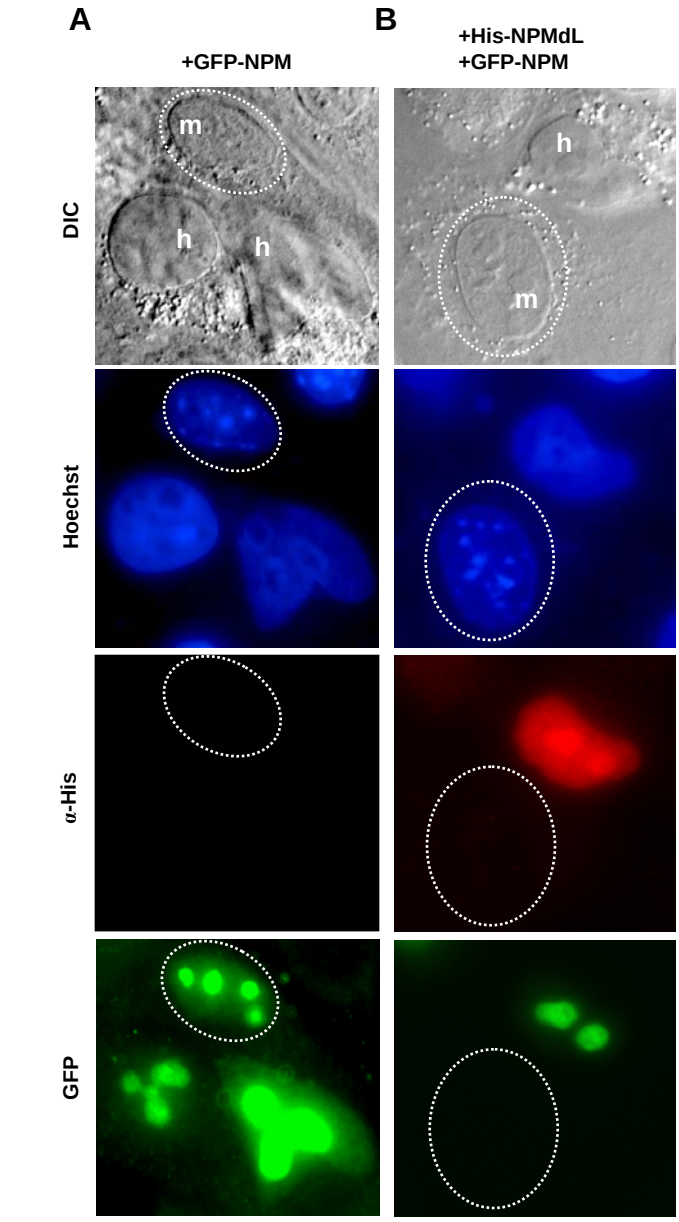
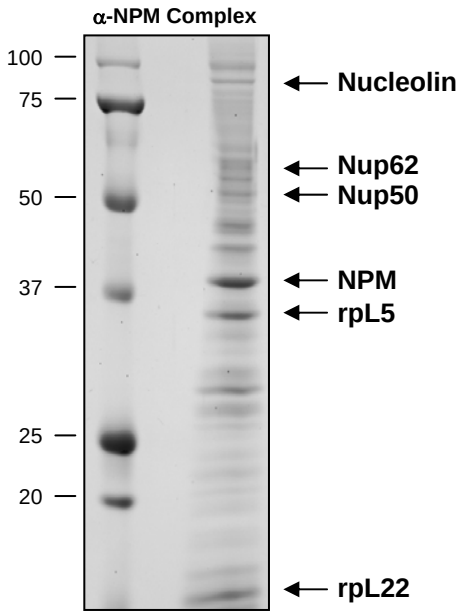
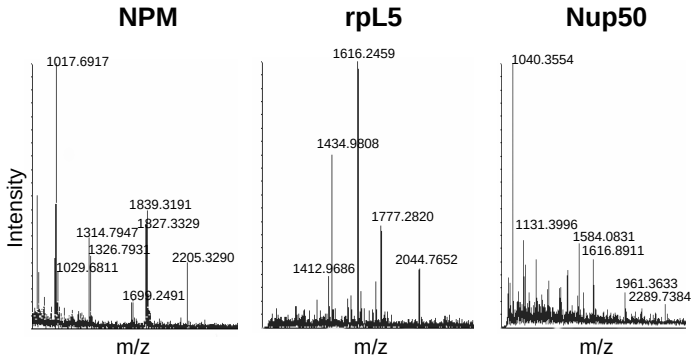

A
B
+GFP-NPM
+His-NPMdL
+GFP-NPM
DIC
Hoechst
α-His
GFP
m
h
h
h
m
GFP-NPM Shuttling: 100%
4%
C
+ His-NPMdL
IP:
—
NRS
His
IB: α-NPM
His-NPMdL
NPM

Figure 4

A


α-NPM Complex
100
75
50
37
25
20
Nucleolin
Nup62
Nup50
NPM
rpL5
rpL22

B


NPM
rpL5
Nup50
Intensity
1017.6917
1839.3191
1827.3329
1314.7947
1326.7931
1029.6811
2205.3290
1699.2491
1616.2459
1434.9808
1777.2820
1412.9686
2044.7652
1040.3554
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m/z
m/z
m/z

C

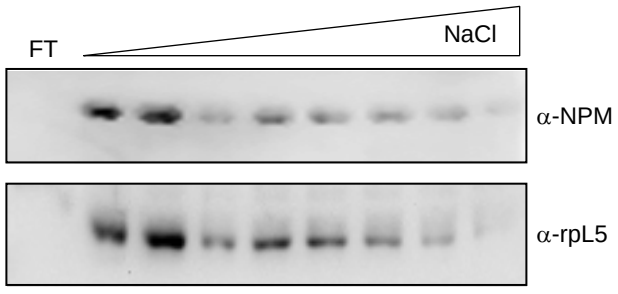
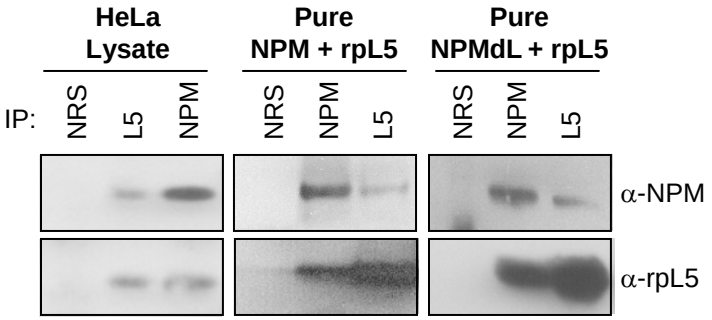
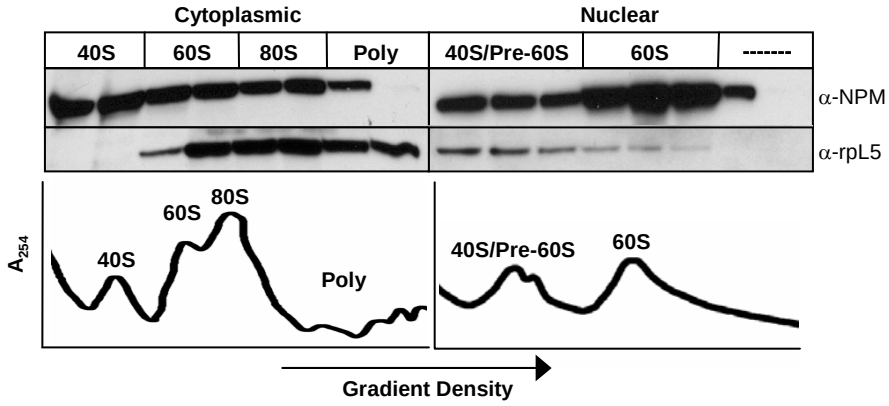

FT
NaCl
α-NPM
α-rpL5

Figure 5

A


HeLa Lysate
Pure NPM + rpL5
Pure NPMdL + rpL5
IP:
NRS
L5
NPM
NRS
NPM
L5
NRS
NPM
L5
α-NPM
α-rpL5

B


Cytoplasmic
Nuclear
40S
60S
80S
Poly
40S/Pre-60S
60S

α-NPM
α-rpL5
A254
40S
60S
80S
Poly
40S/Pre-60S
60S
Gradient Density

Figure 6

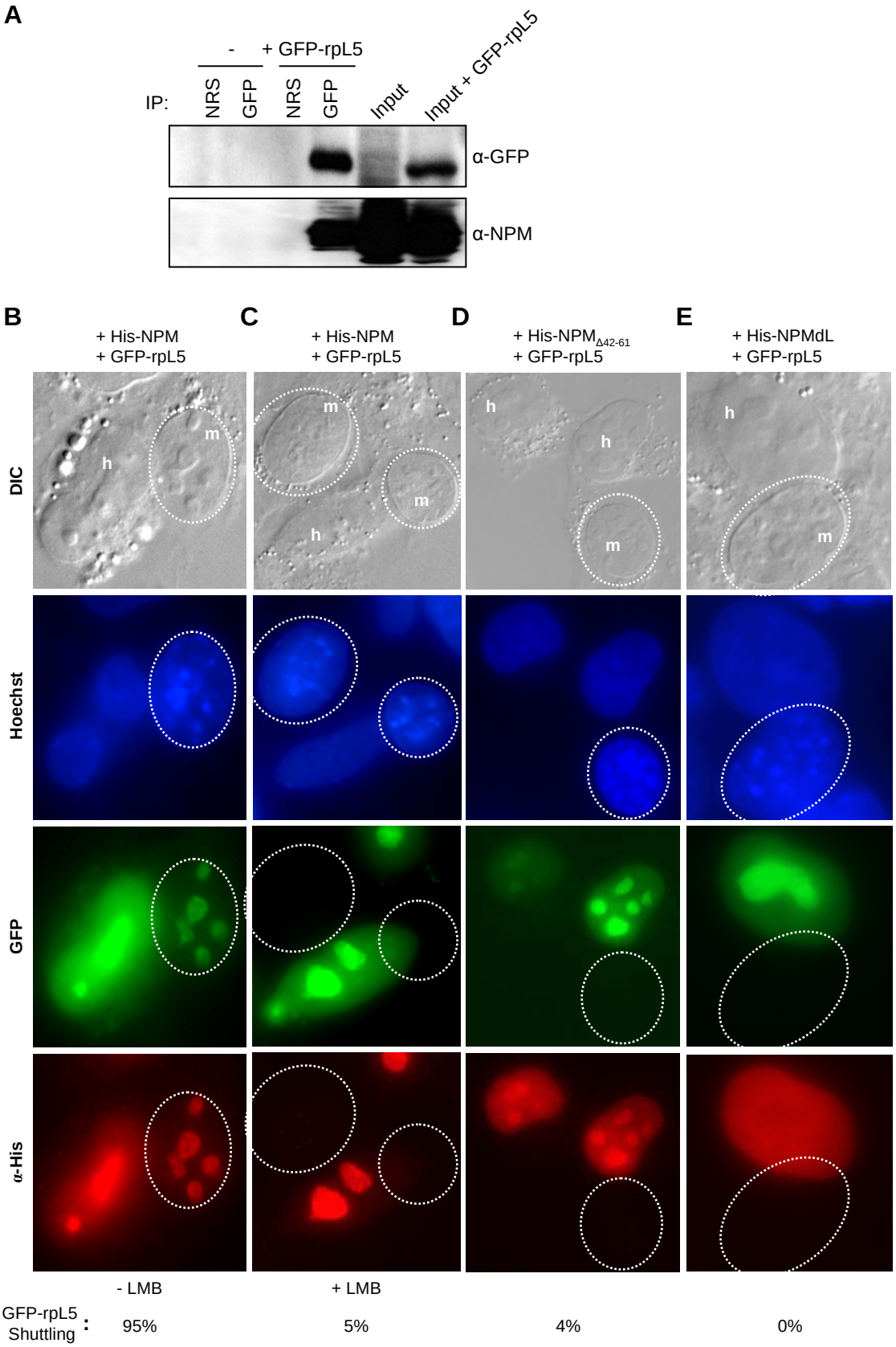
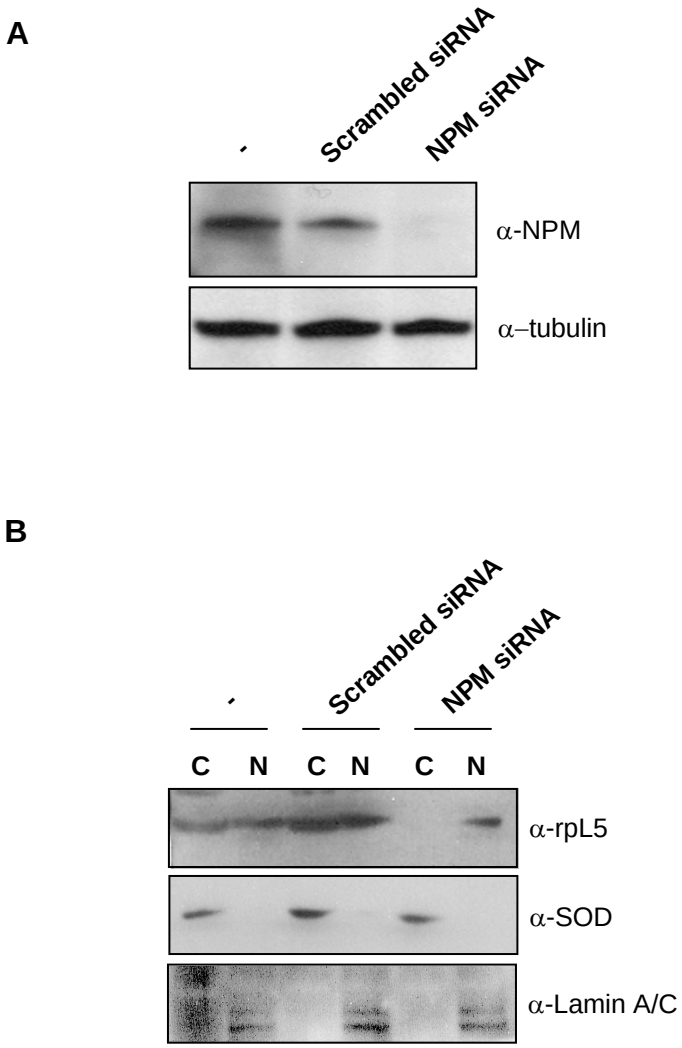

A
- + GFP-rpL5
IP: NRS GFP NRS GFP Input Input + GFP-rpL5
α-GFP
α-NPM
B
+ His-NPM
+ GFP-rpL5
C
+ His-NPM
+ GFP-rpL5
D
+ His-NPM$_{\Delta 42-61}$
+ GFP-rpL5
E
+ His-NPMdL
+ GFP-rpL5
DIC
Hoechst
GFP
α-His
h
m
- LMB
+ LMB
GFP-rpL5 Shuttling :
95%
5%
4%
0%

Figure 7


A
-
Scrambled siRNA
NPM siRNA
α-NPM
α–tubulin
B
-
Scrambled siRNA
NPM siRNA
C N C N C N
α-rpL5
α-SOD
α-Lamin A/C

Curriculum Vitae

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1999-present	Washington University School of Medicine Distinguished Student Scholar (DSS)- a merit-based full scholarship, 1999-2002 MD-PhD candidate**,** Medical Scientist Training Program 2002-present

Honors and Awards:
National Merit Scholar, 1994
Robert C. Byrd Scholar, 1994
Military Order of the Purple Heart National Merit Scholarship, 1994
National Football Foundation & College Hall of Fame Scholar-Leader-Athlete, 1994
New Jersey Commission on Cancer Research Fellow, 1998
George Khoury Award for the Highest Departmental GPA in Molecular Biology,1999
Phi Beta Kappa, June 1999
Alvin J. Siteman Cancer Center Summer Research Fellow, 2000
Howard Hughes Medical Institute Medical Student Fellow, 2001

Teaching Experience:
Undergraduate tutor, 1996-1999
Kaplan Test Prep, MCAT instructor, 1999-2000
Medical school tutor, 2000-present
Teaching Assistant- First-year medical school Physiology course, 2002-2003

Publications:

1. Uhlmann EJ, **Apicelli AJ**, Baldwin RL, Burke SP, Bajenaru ML, Onda H, Kwiatkowski D, Gutmann DH. Heterozygosity for the tuberous sclerosis complex (TSC) gene products results in increased astrocyte numbers and decreased p27-Kip1 expression in TSC2+/- cells. Oncogene 2002 21(25):4050-9

2. Woods SA, Marmor E, Feldkamp MM, Lau N, **Apicelli AJ**, Boss G, et al. Aberrant G-protein Signaling in Nervous System Tumors. J Neurosurg 2002 97(3):627-642.

3. **Apicelli AJ**, Uhlmann EJ, Baldwin RL, Ding H, Nagy A, Guha A, et al. Role of the Rap1 GTPase in astrocyte growth regulation. Glia 2003 42(3):225-234.

Abstracts:

The Role of Rap1 in Astrocyte Growth Control. **Apicelli A**, Uhlmann E, Baldwin R, Lau N, Guha A, Gutmann DH. 2002 Gordon Cancer Conference, poster presentation

Talks:

The Role of Rap1 in Astrocyte Growth Control. 2002 Howard Hughes Medical Student Fellows Meeting

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